

# Plant Genome

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**Biodiversity and Evolution**

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**Volume 1, Part D**  
**PHANEROGAMS**  
**(Gymnosperm) and**  
**(Angiosperm-Monocotyledons)**

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**Editors: A.K. Sharma □ Archana Sharma**

# **PLANT GENOME: BIODIVERSITY AND EVOLUTION**

Volume 1, Part D

**Phanerogams (Gymnosperm) and  
(Angiosperm-Monocotyledons)**

# Plant Genome

## Biodiversity and Evolution

*Series Editors*

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2, Part A : **Lower Groups**  
2, Part B : **Lower Groups**

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A.K. SHARMA and ARCHANA SHARMA



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## **Preface to the Series “Plant Genome”**

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The term *genome*, the basic gene complement of an individual, is almost synonymous with the chromosome complement of both nucleus and organelles. Refinements in cellular, genetic and molecular methods in recent years have opened up unexplored avenues in genome research. The modern tools of gene and genome analyses, coupled with analysis of finer segments of gene sequences in chromosomes utilizing molecular hybridization, are now applied on a wider scale in different groups of plants, ranging from algae to angiosperms. This synergistic approach has made the study of biodiversity highly fascinating, permitting a deep insight into the molecular basis of genetic diversity. Simultaneous to the enrichment of fundamentals in systematics and phylogeny, the plant system, because of its inherent flexibility, has permitted genetic engineering and horizontal transfer of genes with immense importance in agriculture, horticulture and medicine.

Despite the fact that the data on plant genomics with its impact on the assessment of biodiversity and evolution show a logarithmic increase, a comprehensive series on the aspect covering all groups of plant kingdom is sadly lacking. In view of this lacuna, the present series on Plant Genomics: Biodiversity and Evolution has been planned. It aims to cover, in successive volumes, *comprehensive reviews, concepts and discussions on the results of genome analysis and their impact on systematics, taxonomy, phylogeny and evolution of all plant groups*. We have not gone out

of our way to seek original articles, but in course of reviews and discussions, research articles, if any, are welcome.

**A.K. Sharma**  
**Archana Sharma**  
*Series Editors*



## Preface to this Volume

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The coverage of this volume is rather wide ranging from Gymnosperms with the two important groups the Cycads and the Pines, to monocotyledons represented by genera having special significance in terms of phylogeny, agriculture, horticulture and commercial importance. The dicotyledonous genera have not been included as the preceding volume I C dealt exclusively with dicotyledons.

In gymnosperms, the DNA sequence data have aided in ascertaining the uniqueness of Cycads as distinct from *Ginkgo*, *Gnetum* and Pines. The phylogenetic relationship within Cycadophytes has been studied from chloroplastid sequences and the monophyletic nature of *Cycas*, basic to Encephalarteae and Zamieae has been clearly indicated. On the other hand, in *Pinus* a correlation between genomic diversity and microhabitat characteristics has been recorded suggesting that climatic selection and stress have played a prominent role in adaptation of genetic variants.

In the monocotyledons, RAPD, ISSR and SSR have been employed in establishing diversity in *Phoenix dactylifera*, the oil palm, simultaneously bringing out evidence of a common genetic basis characterizing ecotypes of this species. The significant finding in date palm on the other hand, is the single gene control of shell thickness and the origin of all products of commercial value through hybridization of *dura* mother and *pisifera* pollen.

In *Allium*, along with other approaches, for analyzing genome complexity, the use of molecular markers in determining trends of diversification, hybridity, genetic map and genome analysis has been



recorded. Detailed chromosome and *in situ* hybridization as reported has been presented.

Of the two reviews on Orchidaceae, one deals exclusively with phylogeny and evolution in *Cymbidium* as ascertained through nrITS and RAPD data. Molecular data shows partial congruence with current taxonomy and ITS and plastid matK could delineate only a few subgenera. The other review deals with genome evolution and population biology in the family as a whole. The results of study with an array of unclear and organellar markers indicate distinctive features of terrestrial, epiphytic and lithophytic orchids.

Of the grasses, cytological and molecular characteristics along with morphology have been utilized in determining phylogeny, affinities and status in *Bromus*. Moreover allozyme pattern and cpDNA and rDNA have aided in highlighting controversial issues in this genus.

In the subgroup Loliineae, cytogenetic, morphoanatomical and morphological data in *Festuca* in particular have been taken to suggest that evolution of broad leaf, large genomes with low heterochromatin have led to fine leaved taxa with small genome and high heterochromatin. Simultaneously, polyploidy and hybridization have led to the evolution of Annual from Perennial habit. Discrepancies in phylogeny reconstruction between morphological and molecular data have been recorded though karyotype evolution in subtribe Loliineae which is concordant with molecular data.

Finally, in *Avena*, the oats, cytological, genetic and molecular data specially including two sat DNA sequences As12O a and Am 1 have been utilized to identify the genomes of a new tetraploid species. The putative progenitors of the hexaploid AACCCD have also been delineated indicating steps in their evolution. This volume on plants of economic importance and of phylogenetic value would be an asset to anyone interested in genomics and evolution of plants of commercial and agricultural importance.

July 28, 2006

A.K. Sharma  
A. Sharma

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# Evolution and Phylogeny of Cycads

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## ABSTRACT

Cycads represent a monophyletic plant group that is traditionally grouped in 3 families and 11 genera with a total of 250 species. DNA sequence data suggest that extant cycads evolved during the last 60 million years and they represent a unique group besides *Ginkgo*, Gnetophyta and Pinophyta. Sequences from *cpDNA* (chloroplast DNA) are used to determine the phylogenetic relationship within the Cycadophyta. The genus *Cycas* is monophyletic and clusters basal to the other cycad genera. *Encephalartos*, *Macrozamia* and *Lepidozamia* form a well recognized clade, that is commonly included in the tribe Encephalarteae. *Zamia*, *Chigua* and *Microcycas* also form a well-supported clade, recognized in the tribe Zamieae. The position of *Ceratozamia*, *Dioon* and the almost monotypic genera *Stangeria* and *Bowenia* cannot be resolved unambiguously with the present data sets. No evidence could be found for a family Stangeriaceae that includes both *Bowenia* and *Stangeria*.

A molecular clock approach is employed to estimate the age of extant cycads. The Cycadaceae/Zamiaceae split may have occurred about 50 million years ago (mya). The African-Australian disjunction of *Encephalartos*, *Lepidozamia*, and *Macrozamia* is much younger and can be explained by long-distance dispersal across the ocean in the Miocene rather than by continental drift. The extant species of *Encephalartos* evolved about 6.5 mya and spread over Southern and Central Africa in the late Pleistocene and Pliocene (5-1.6 mya). The colonization of Madagascar by *Cycas thouarsii*, far from the distribution areas of the other *Cycas* species, seems to be very recent and might probably even be due to

human intervention. Also, the colonization of several islands in the Pacific and Indian oceans by other members of the *Cycas* subgroup Rumphiae is apparently correlated with the presence of the spongy endocarp that allows seeds of these species to be transported via the sea.

**Key Words:** Cycads, molecular phylogeny, gymnosperms, molecular clock, long-distance dispersal, Gondwana

**Abbreviations:** ML= Maximum likelihood, MP= Maximum parsimony, NJ= Neighbour-joining.

## INTRODUCTION

Cycads, also known as palm ferns, represent a small group of slow-growing woody perennials with slow recruitment and population turnover rates that are united by several unique characters [12, 22]. As they have large divided leaves, the cycads resemble tree ferns or palms to some degree. Cycads generally have pinnate leaves that are spirally arranged in crowns on the stem apex and pubescent when young. Another characteristic trait is the pachycaul stem consisting of storage tissue rich in starch. Cycads are dioecious and reproduce by seeds that are produced on open carpophylls or seed-bearing leaves. Except for Cycadaceae, sporophylls are arranged into cones. Male plants carry several sporangia on the under- or abaxial surface of the sporophylls. Pollen is released from slits in the sporangia. Male gametophytes have several flagellae and are motile. Female sporophylls produce large seeds with a two-layered testa; the outer layer is often coloured and fleshy, which serves to attract animals. The animals such as birds, rodents small marsupials and fruit-eating bats eat the seeds, thereby help in seed dispersal. Another unique trait is coralloid and contractile roots that occur in addition to normal roots. The coralloid roots harbour nitrogen fixing symbiotic Cyanobacteria, which allow cycads to live on soils that are poor in nutrients—ranging from dense tropical forests to semideserts in tropical or subtropical climates with summer rainfall.

Cycads have naked ovules, a character shared with other “gymnosperms” such as the *Welwitschia* group (Gnetophyta), the conifers (Pinophyta) and *Ginkgo* (Ginkgophyta). All “gymnosperms” represent ancient (arising in the Permian era) seed plants, of which many are now extinct, except for the four major extant groups. Molecular evidence for the relationships within these basal groups is elaborated in this chapter.

Presently more than 250 species are recognized in the order Cycadales in the class Cycadophyta, grouped in 11 genera, which make

them a small group within the flowering plants with more than 300,000 species [7, 8, 23]. Figure 1 shows an overview of the classification of cycads into 3 families, 4 subfamilies, and 11 genera [23]. A concise history of cycad taxonomy is given by Hill et al. [7].

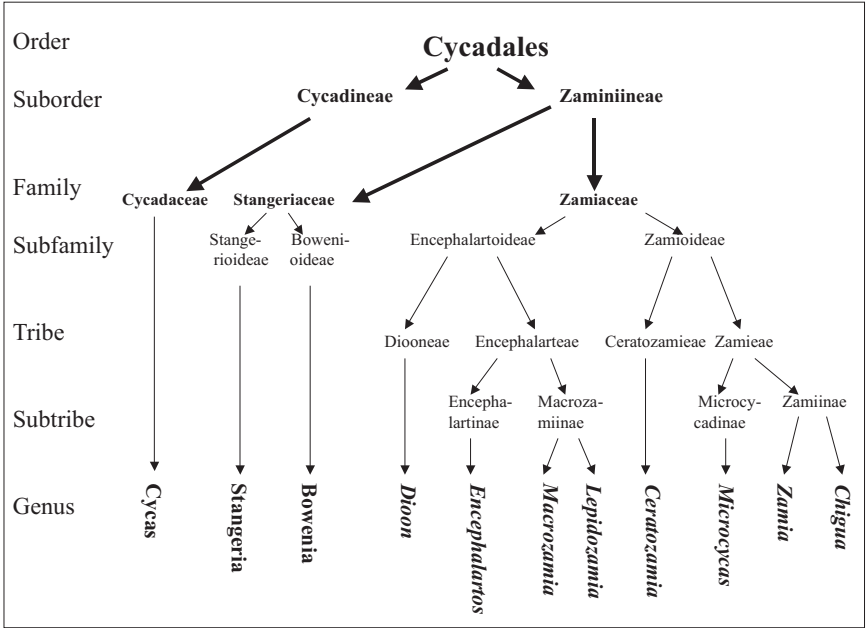


Fig. 1. Classification of cycads.

The cycad origins go back to the Early Permian era, about 280 mya; it has been suggested that they evolved as a sister-group to all other seed plants. Their ancestors could have been the more ancient (i.e. Palaeozoic) seed ferns. The cycads had their maximum expansion during the Triassic to Tertiary era. In these times, cycads were widely distributed [12], the Jurassic period, is often termed as the ‘Age of Cycads’. Cycads are often called living fossils because their overall morphology has changed negligibly from their ancestors in Mesozoic times. However, the three extant families of today show similarities only to fossils from Tertiary about 50-60 mya, whereas more than 19 extinct cycad genera are only known as fossils of the Palaeozoic and Mesozoic era [12]. As described in this review, cycads did not stop their phylogenetic

development 200 mya ago but underwent phylogenetic speciation as any other plant order. The consequence is that species living today may, in fact, be quite young [24, 25]. Fossil cycads have been detected in Mesozoic deposits of every continent and every latitude, ranging from Siberia to the Antarctic. Cycads are now only found sparsely distributed in the tropics and subtropics (Fig. 2) of the formerly united, but later separated supercontinents Laurasia and Gondwana. They are now reduced in numbers and distribution. It would be plausible to explain the extant distribution in terms of plate tectonics, but as discussed later in this review, also long-distance dispersal via the sea was another mechanism for the present distribution of cycads.

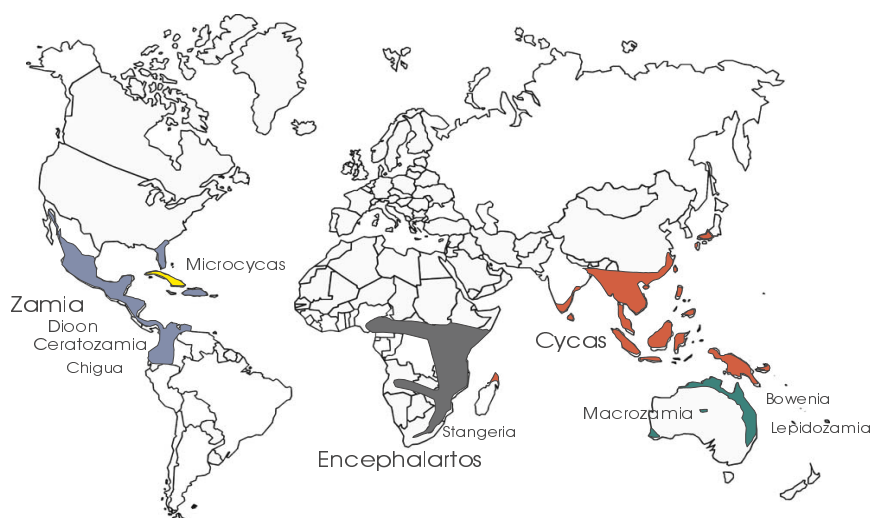


Fig. 2. Present day distribution of cycads.

## SECONDARY METABOLITES IN CYCADS

Cycads produce a series of unique secondary metabolites, as well as dimeric flavones and the nitrogen containing methylazoglycosides cycasin, and macrozamin with effective repellent and toxic properties [2, 20]. There is some direct and additional circumstantial evidence that the cycasins are the central protective means of cycads against herbivores. The effective, toxic component of these glycosides is their aglycon, methylazomethanol (MAM). MAM is a methylating agent that

can covalently modify DNA. It is, therefore, mutagenic and potentially carcinogenic. The azoglycoside levels in cycads range from 0.01 to 5% of fr. wt [11, 20].

Another cycad toxin is a non-protein amino acid  **$\beta$ -N-methylamino-L-alanine** (BMAA) [2], which in higher concentrations was found to be neurotoxic for mammals and chickens, and also suspected to be the cause of neurological syndromes in man. BMAA occurs, like cycasin, in nearly all cycad genera analysed so far, yet with a wide range of genus-specific, quantitative differences. BMAA (fr. wt.) contents range below those of the azoglycosides: leaves and seeds 0.0002-0.17%: *Zamia* cone tissue 0.001%; cone storage cells (ideoblasts) 0.04% [20]. The ubiquitous occurrence of cycasin and BMAA in all extant cycad genera strongly suggests that these defence compounds evolved early in the history of these plants [12, 20].

Cycads are largely avoided by herbivores, yet some insects are adapted to cycads and so are some vertebrates which eat cycad leaves and fruits. These insects are (besides some aphids, thrips and scale insects), leaf-eating larvae of Lepidoptera and Coleoptera of several families [20] and play a role in the pollination of cycads [20]. The cycads have been generally thought to be wind pollinated. However, several recent studies in different regions indicate that cycads are mostly insect pollinated, often by weevils that are closely dependent on the cycads. This contrasts with both *Ginkgo* and the conifers (the other primitive seed plants), all of which are wind pollinated [14]. Chemistry of the pollinator-attractants in cycads is markedly different from that of other flowering plants, suggesting that insect pollination has evolved independently in the two groups. Species of several beetle taxa were observed in a variety of interactions with the cycads, reaching from plain herbivory to symbiotic, mutualistic pollination. Curculionid beetles of several families represent a majority of the associations with cycads. The currently best understood cases of such mutualism are species-specific relations between two New World cycads and two snout-weevils in Florida. The first pair comprises the endemic cycad *Zamia integrifolia* (= *Z. pumila*) and its symbiotically colonizing weevil *Rhopalotria slossonae* (Belidae) and the second pair consists of the originally Mexican *Z. furfuracea* and its also introduced partner *R. mollis*. Interestingly, another beetle, *Pharaxonothus zamiae* (Languriidae) is an additional pollinator/parasite of the Floridan *Zamia integrifolia* ([12, 20].

## MOLECULAR PHYLOGENY OF CYCADS

### Phylogenetic Position of Cycadophyta

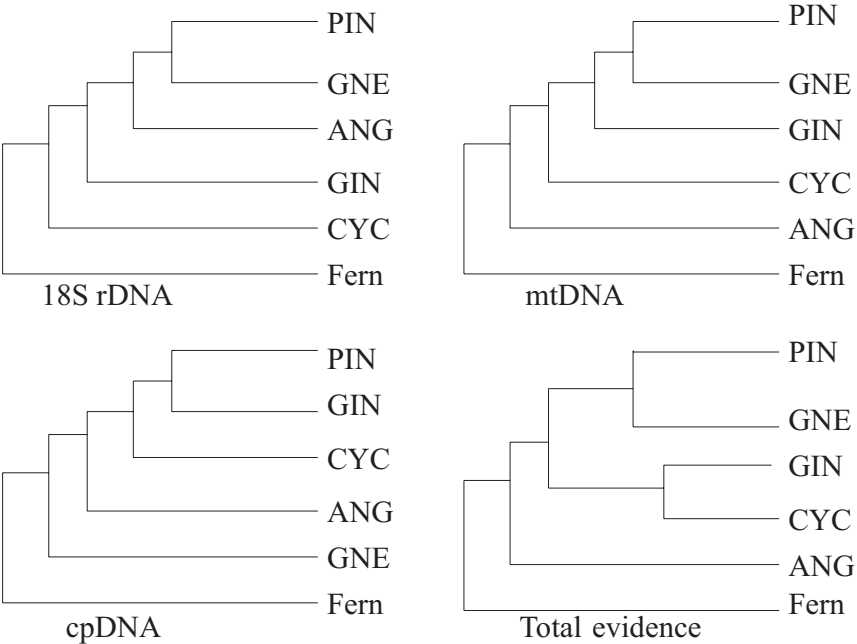
A number of research groups have used DNA sequences of cpDNA, mtDNA (mitochondrial DNA), nuclear ITS (nuclear ribosomal DNA internal transcribed spacer) or 16S and 18S rDNA to elucidate the evolutionary position of cycads [1, 3, 15, 21, 24, 25]. A summary of the results obtained from different marker genes is shown in Fig. 3. It is apparent that the phylogenetic position of cycads strongly depends on the marker genes that have been analyzed.

Ribosomal genes place cycads at the base of the tree leading to seed plants. Since rDNA-genes were the first ones to be analyzed, the view that cycads are basal to the rest of the seed plants derives from the early days of molecular systematics. Subsequent studies on chloroplast genes changed the overall topology and placed the Gnetophyta at the basal position, followed by a bifurcation that split angiosperms and the rest of the “gymnosperms”. Cycads are basal to the residual gymnosperms and clusters as a sister to *Ginkgo* and conifers (Fig. 3). Phytochrome genes also revealed a similar topology [19]. Mitochondrial genes that have been somewhat neglected in plant phylogeny, reveal angiosperms as a basal clade followed by cycads that is basal to *Ginkgo*, conifers and Gnetophyta. Soltis et al. [21] have combined the three data set to form one combined data set. Here, similar to the situation in the mtDNA data set, the angiosperms are basal, followed by a monophyletic gymnosperm cluster that is divided into a cycad/*Ginkgo* and Gnetophyta/conifer clade.

Since Cycads share the plesiomorphic character “naked ovules” with other “gymnosperms” such as the *Welwitschia* group (Gnetophyta), the conifers (Pinophyta) and *Ginkgo* a (Ginkgophyta), the results of the mtDNA and combined data set have the advantage that they concur with the traditional perspective that gymnosperms and angiosperms are monophyletic assemblages.

### Phylogenetic Structure within the Cycadophyta

DNA sequences of marker genes can also be used to elucidate the phylogenetic relationships within the monophyletic Cycad ensemble. The following discussions are mainly based on results of my own laboratory [24, 25, 26, 27] and those of [7].



**Fig. 3.** Phylogenetic position of Cycadales among gymnosperms and angiosperms based on nucleotide sequences of different sets of marker genes.

GNE= Gnetophyta, ANG= Angiospermae, CYC= Cycadophyta, GIN = Ginkgophyta, PIN= Pinophyta

For the total evidence trees, the data from 18SrDNA, mtDNA and cpDNA were combined (from Soltis et al. [21]).

Figures 4A, B and C show a phylogenetic reconstruction based on *rbcL* (RuBisCo large subunit gene) sequences of all cycad genera, *Ginkgo* and Gnetales and selected genera from conifers, in comparison to some mono- and dicots. These main groups cluster as expected and are known from other studies [1, 3, 15, 21, 24] as monophyletic clades. The Gnetophyta are always at the base of the clade leading to higher plants (bootstrap support 100%). The next bifurcation divides branches leading to angiosperms (with mono- and dicots) and residual gymnosperms (bootstrap support 98%). Within the residual gymnosperms, conifers (Pinophyta) cluster as a sister to Ginkgophyta and Cycadophyta, which cluster as a terminal sister pair (bootstrap support 99-100%). These relationships are always recovered independent from the method of phylogeny reconstruction (i.e. MP, NJ, ML). Almost identical topology



A

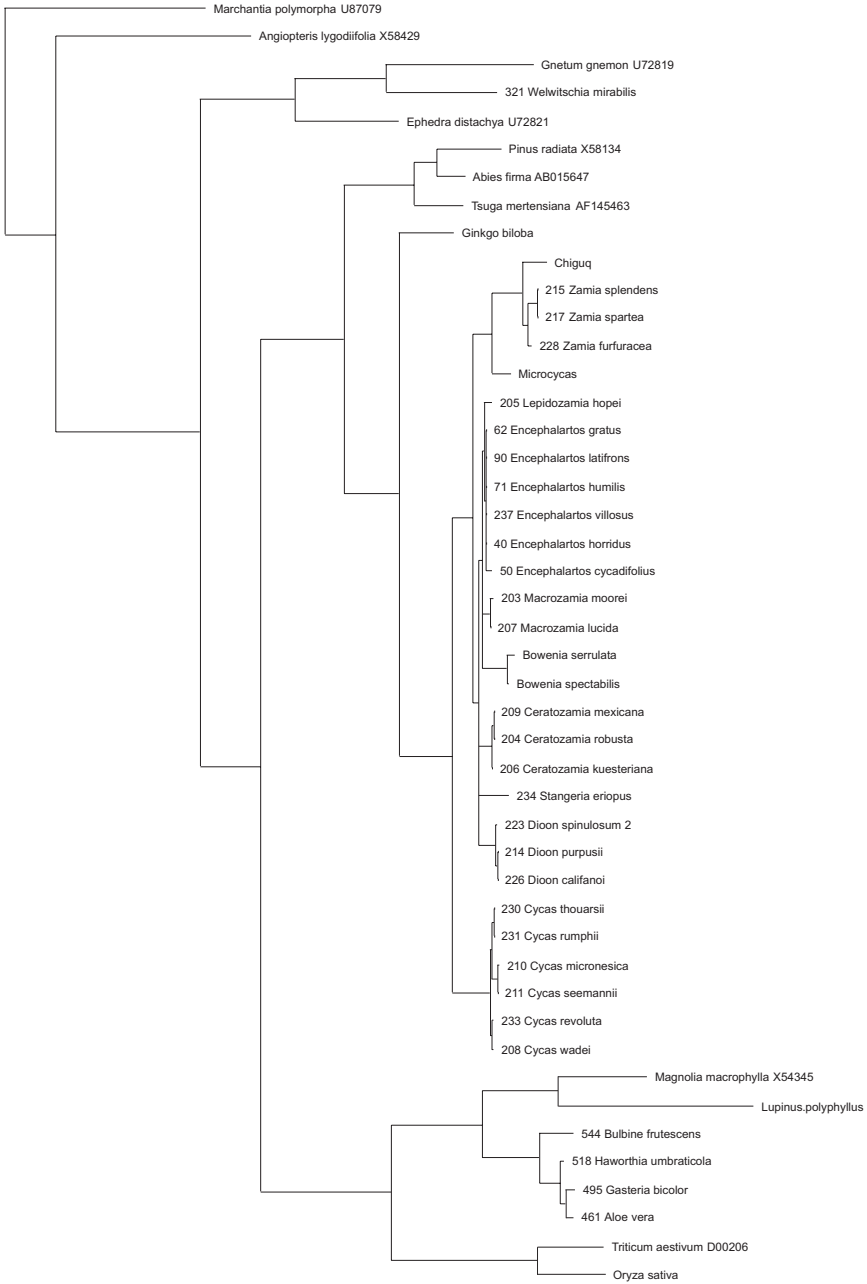


Fig. 4 contd.

B

Strict

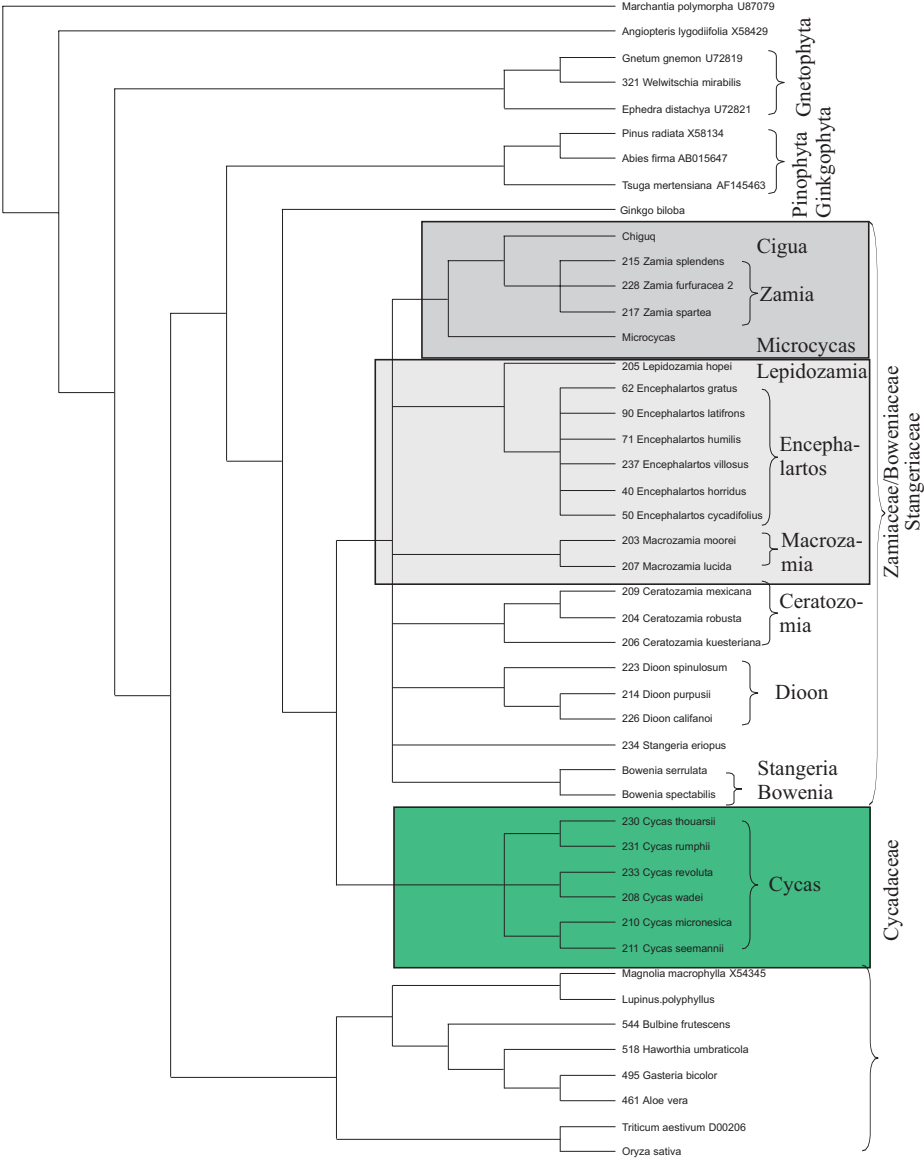
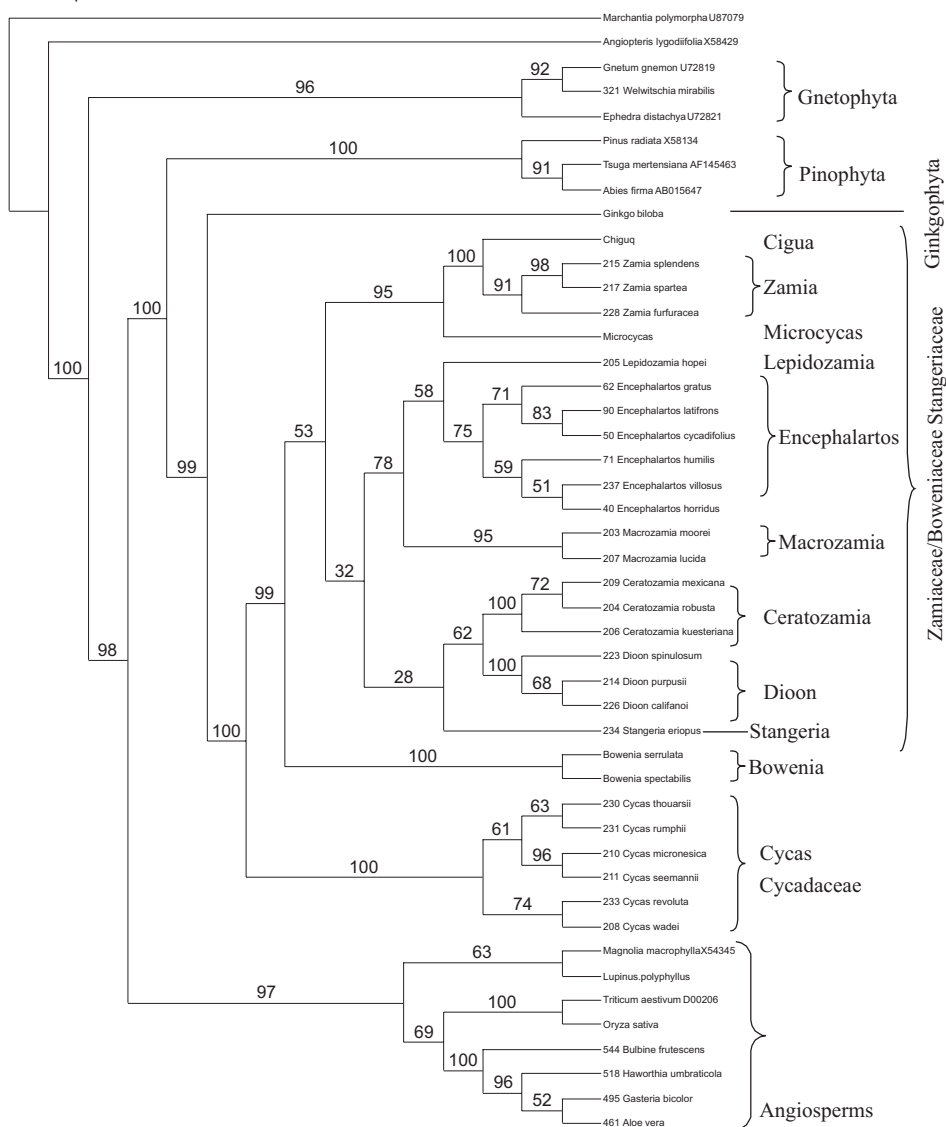


Fig. 4 contd.

## C

Bootstrap



**Fig. 4.** Phylogenetic relationships within cycads based on sequences of the *rbcL* gene. A liver moss (*Marchantia*) and a fern (*Angiopteris*) were selected as far distant outgroups. Tree length: 167 steps; CI=0.554, RI= 0.739; RC= 0.409, HI= 0.446; of 1,428 characters 408 are parsimony informative. A. One of 270 most parsimonious trees; B: MP strict consensus; C: NJ with Kimura 2 parameter as distance algorithm; bootstrap values (1,000 replications) are shown above branches.

had been recovered by Soltis et al. [21] and especially Rai et al. [15], who had employed sequences of 17 chloroplast genes and associated non-coding regions.

The phylogenetic trees (Figs. 4A, B and C) also illustrate the relationships within the Cycadales. Members of particular cycad genera cluster as monophyletic clades. Among them, *Cycas* forms the most basal group (100% bootstrap support), justifying its treatment as a separate family Cycadaceae. This is in agreement with previous morphological studies [9, 12, 23] suggesting that the *rbcl* phylogram reflects the true phylogeny, even if the full picture of species phylogeny cannot be concluded solely from a single gene. However, *Cycas* had a basal position also in an analysis involving sequences from cpDNA, ITS and 26S rDNA [7].

More complicated is the situation with the two other cycad families, the large Zamiaceae and the very small Stangeriaceae (with only three species).

Both Cycadaceae and Zamiaceae form well-supported clades. *Stangeria* clusters within Zamiaceae, which questions its systematic status as a monotypic family. *Encephalartos*, *Macrozamia* and *Lepidozamia* form a well recognized clade (Fig. 4C) that is commonly included in the tribe Encephalarteae. This finding is corroborated by an analysis involving sequences from cpDNA, ITS and 26S rDNA [7]. *Zamia*, *Chigua* and *Microcycas* also form a well-supported clade, recognized in the tribe Zamieae. This finding is also supported by an analysis involving sequences from cpDNA, ITS and 26S rDNA [7].

The position of *Ceratozamia*, *Dioon* and the almost monotypic genera *Stangeria* and *Bowenia* cannot be resolved unambiguously with the present data set. However, an analysis involving sequences from cpDNA, ITS and 26S rDNA [7] implied that *Ceratozamia* might be a sister to the tribe Zamieae. *Dioon* clusters at the base of a clade including the tribe Encephalarteae and the genus *Bowenia*. These phylogenetic relationships are, however, only weakly supported by bootstrap values. According to [7], *Stangeria* takes a basal position of the clade comprising the Zamiaceae and *Bowenia*. No support was found for a family Stangeriaceae (sensu Stevenson [23]) that includes the genera *Bowenia* and *Stangeria*. In conclusion, the molecular data are in conflict with all previously proposed classifications of the Cycadophyta at one or more levels. More work is clearly needed to understand the relationships of *Stangeria*,

*Bowenia* and *Dioon* in order to establish a natural classification of Cycadophyta.

## **Phylogenetic Relationships within the Genus *Encephalartos***

*Encephalartos*, which is the largest genus of the Cycadales comprising 55 species [6, 12], is geographically restricted to Africa (south of the Sahara). A significant number of species occur in the tropical regions of central and east Africa, but more than half in South Africa. They occupy a wide range of climatic regimes and habitats, and show a high degree of endemism [4]. Research on relationships between these taxa has been done at a morphological and biochemical level [26, 27, 28, 29], but nucleotide sequencing has been applied only to a few taxa [24, 25, 26, 27].

*rbcL* sequences (Fig. 4) clearly indicate that the genus *Encephalartos* forms a monophyletic clade with *Lepidozamia* and *Macrozamia* [7, 24]. Nuclear internal transcribed spacer regions (ITS 1 and 2), have been used to resolve the phylogenetic history of the genus *Encephalartos*; the chloroplast encoded *rbcL* gene was of little use since only few nucleotide positions differ between species (Table 2) [25].

ITS 1 and 2 resolve *Encephalartos* in three distinct clusters (indicated as ITS-groups 1, 2, 3 and their subgroups, respectively) (Fig. 5) that agree well with the intragenic structure based on morphological and geographical characters (Fig. 6).

MP and ML trees of ITS 1 and 2 are almost congruent in topology, indicating that the phylogenies are well-supported by the sequence data (Fig. 5). The taxa of ITS-group 1 form the most basal group (within-group maximal p-distance: 0.8%) and are also separated from the rest of the taxa by morphological as well as ecological data. The monophyly of group 1 is statistically well-supported by 89% bootstrap value. The branch leading to the sister-group of ITS-group 1 (composed of clusters 2 and 3) is even better supported (bootstrap value of 91%). Taxa of clusters 2 and 3 show a maximal p-distance of 3.7% to cluster 1 and are resolved by 63 & 95% bootstrap, respectively. ITS-cluster 2 consists (maximal 1.7% p-distance) of 6 subgroups and further 5 ungrouped species, and is widely distributed from the Eastern Cape (*E. caffer*) to Uganda (*E. septentrionalis*), Kenya (*E. tegulaneus*) and Nigeria (*E. barkeri allochrous*). Subgroups 2.0, 2.1 and 2.2 are well-supported by 70, 91 and

ITS 1&2  
MP  
Strict

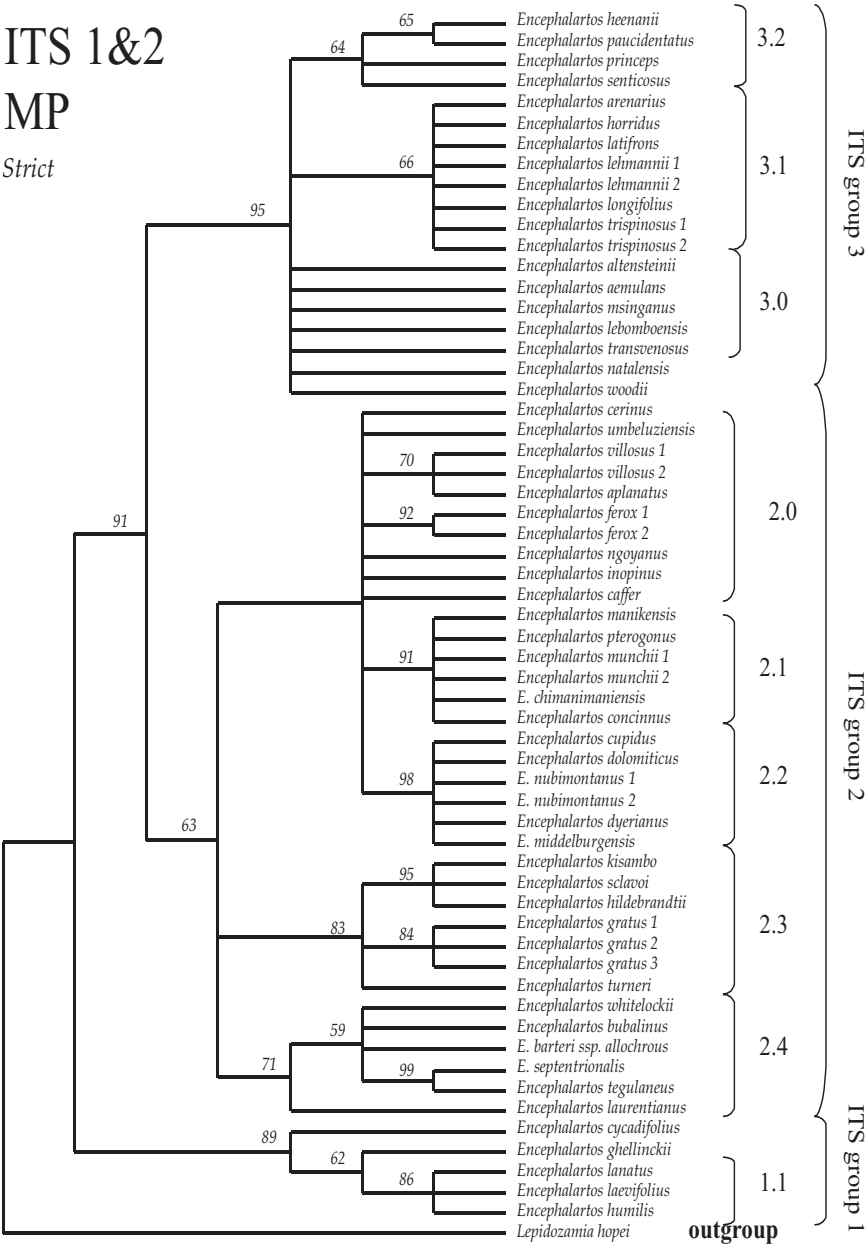


Fig. 5A

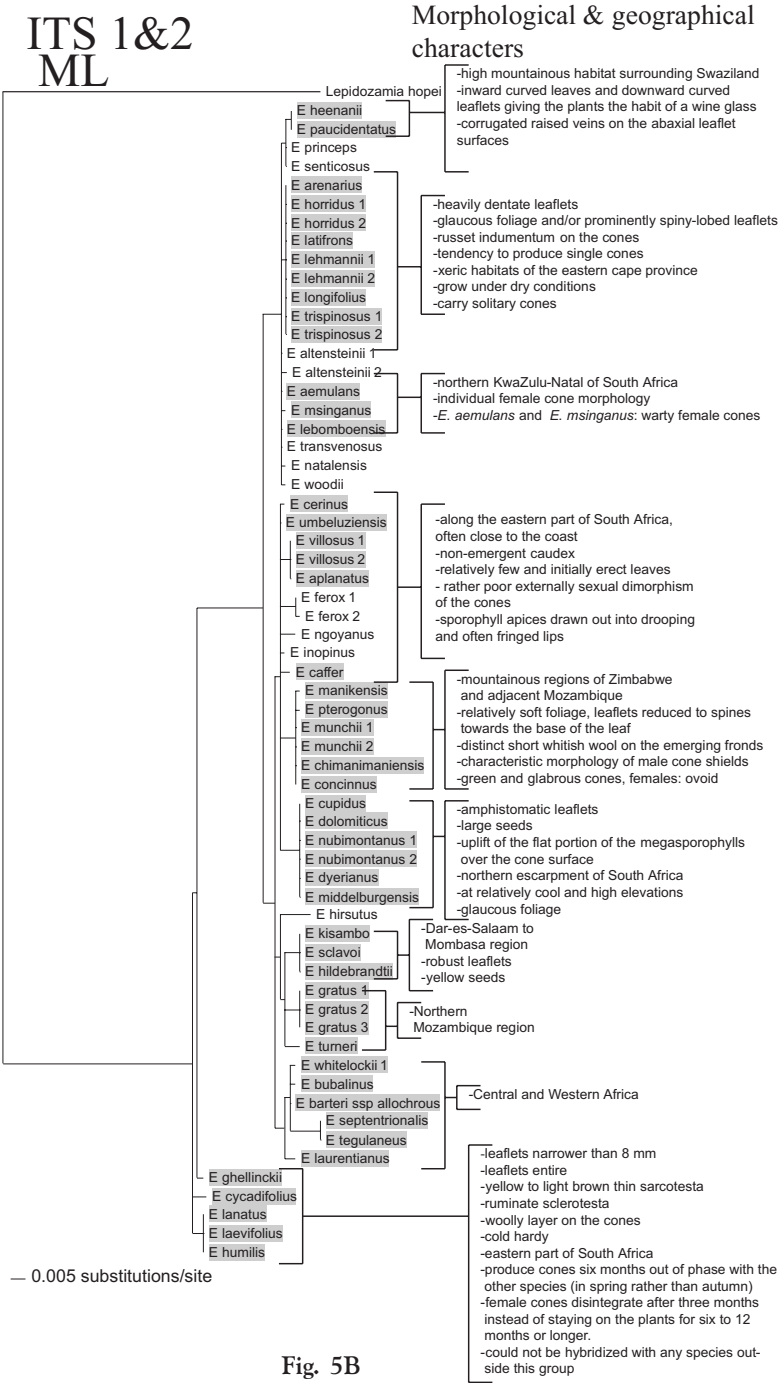


Fig. 5B



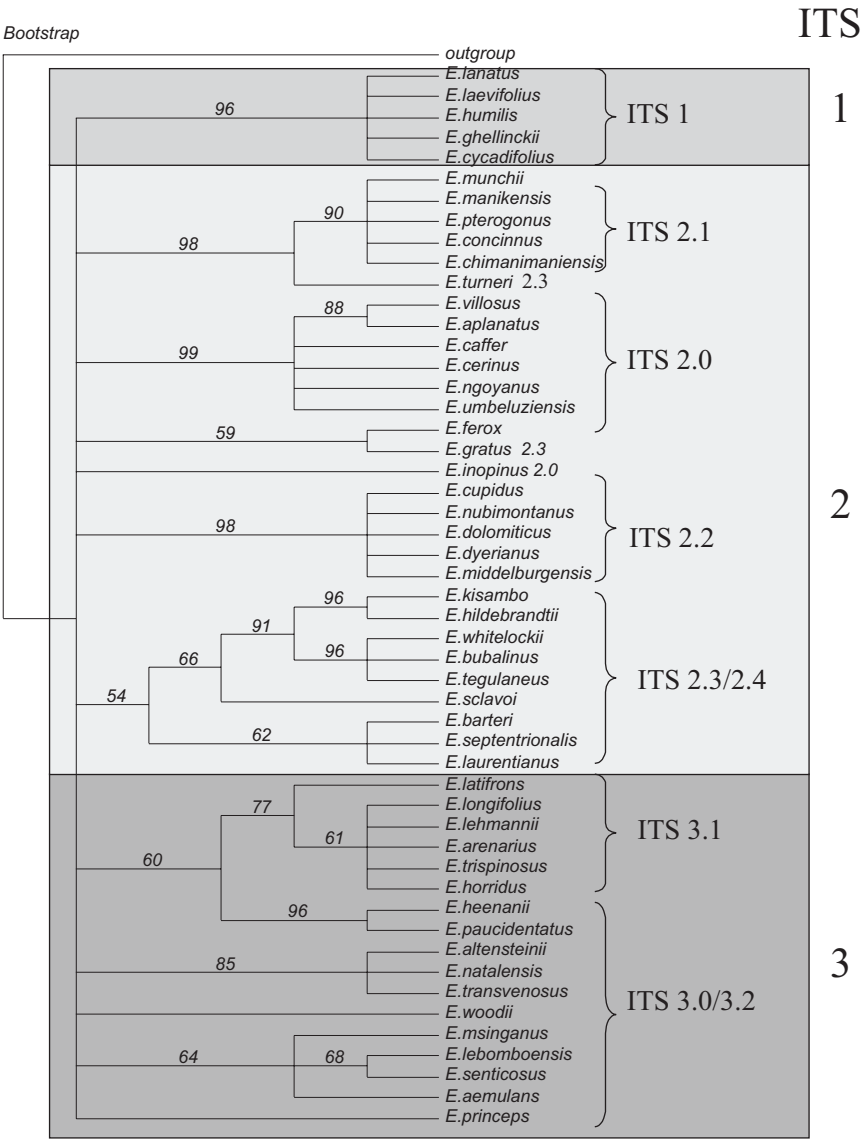
98% bootstrap value, and subgroup 2.3 by 83% bootstrap support. Taxa of subgroup 2.3 are distributed from Mozambique (*E. gratus*) to Kenya (*E. kisambo*), and are internally further structured: *E. sclavoi*, *E. hildebrandtii*, and *E. kisambo* (95% bootstrap support) within subgroup 2.3 grow in the very restricted region of Dar-es-Salaam to Mombasa, and are characterized by leathery leaflets and yellow seeds. Subgroup 2.4 (71% bootstrap support) is the most diverse among all the subgroups and distributed along the equator in Africa from the west (*E. barteri allochrous*) to the east (*E. whitelockii* in Uganda, *E. tegulaneus* in Kenya, and *E. bubalinus* in Tanzania). Consequently, ITS-group 2 is distributed over a wider distribution range than any other group or subgroup. All the taxa of ITS-group 3 (95% bootstrap support, maximal p-distance 0.5%) occur in the area from Willowmore in the south (*E. lehmannii* and *E. longifolius*) to Piggs Peak in Swaziland in the north (*E. paucidentatus*); taxa of subgroup 3.1 usually grow in dry habitats of the Eastern Cape and share glaucous leaflets as a common character. Detailed morphological features and biogeographical affinities are given in Table 1.

A genomic fingerprinting technique was employed using ISSR-PCR (inter simple sequence repeat-polymerase chain reaction) to study the taxonomy of *Encephalartos* [25]. ISSR was able to support only two of the relationships found by ITS 1&2. The group ITS 1.1, consisting of *E. lanatus*, *E. laevifolius* and *E. humilis*, is supported by ISSR-band 4 and in part by ISSR-band 7. Interestingly, the taxa *E. cycadifolius* and *E. ghellinckii*, that clearly belong to ITS-group 1 by ITS 1&2 and morphology, show neither band 4 nor band 7 fragments and are clustered differently by ISSR. A second ISSR group that represents the ITS 1&2

**Fig. 5.** Molecular phylogeny of *Encephalartos* based on nucleotide sequences of ITS I and II [from 25]

A. Strict consensus parsimony cladogram of ITS 1&2 constructed by MP. Parameters: sequence addition: closest; branch swapping option: tree bisection-reconnection. Bootstrap values (1,000 replications) above 50 are presented. 1,152 most parsimonious trees of the length 214 were obtained, of which the strict consensus cladogram is shown here (CI 0.916; RI 0.944; RC 0.865; HI 0.084).

B. Reconstruction by ML. Two trees  $-\ln L = 1798.26007$  were obtained. Results are presented as phylogram with branch lengths representing the genetic distance under the GTR+G+I algorithm. User-specified substitution rate matrix: AC=0.642793 AG=4.437940 AT=1.687732, CG=1.496376, CT=3.590283, GT=1.000000; Assumed proportion of invariable sites = 0.341177; shape parameter (alpha)= 401.87.



**Fig. 6.** Most parsimonious tree of *Encephalartos* morphological and geographical traits [after 25]: from a matrix of 27 morphological and biogeographical characters. Bootstrap values (1,000 replications) above 50 are presented.

Table 1. Morphological and geographical characters of the cycad genera (from [7, 8, 22]), N = numbers of species

Family	Genus	N	Occurrence	Characteristic traits
Cycadaceae	Cycas L.	90	Australian (26 species); Indo-Chinese region (30 species); Malaysian region; Japan; SE Asia; Micronesia; Polynesia; Madagascar; E. Africa	<b>Megasporophylls:</b> not in cones, in a terminal rosette; <b>ovules:</b> 2 or many; <b>seeds:</b> yellow, orange or brown fleshy outer sarcotesta; <b>microsporophylli:</b> numerous microsporangia assembled in determinate male cones; <b>stem:</b> aerial or subterranean, pachycaul & cylindrical; <b>leaves:</b> pinnate or rarely bipinnate; <b>leaflets:</b> midrib, lacking secondary veins; not articulated.
Zamiaceae	Dioon Lindl.	11	Mexico, Honduras & Nicaragua	<b>Megasporophylls:</b> in spirally assembled stalked female cones; ovules: 2 (rarely 3), stalked; <b>microsporophylls:</b> spirally assembled in stalked male cones; <b>seeds:</b> white or cream fleshy outer sarcotesta; <b>stem:</b> aerial or subterranean, pachycaul & cylindrical; <b>leaves:</b> pinnate, spirally arranged; lower leaflets often reduced to spines; <b>leaflets:</b> simple, often with spiny margins & numerous bifurcating parallel veins and without distinct midrib; broad-based, not articulated; <b>basal offsets:</b> common.

Table 1 contd.

Table 1 contd.

Encephalartos Lehm.		62	Africa	<p><b>Megasporophylls:</b> in spirally assembled stalked female cones; ovules: 2 (rarely 3), sessile; <b>microsporophylls:</b> spirally assembled in determinate, stalked or sessile male cones; <b>seeds:</b> with a red, yellow, orange or brown fleshy outer sarcotesta; <b>stem:</b> often large aerial or subterranean, pachycaul &amp; cylindrical, with usually many leaves and persistent leaf bases; <b>leaves:</b> pinnate, spirally arranged, lower leaflets often as spines; <b>leaflets:</b> simple, frequently with spiny, dentate or lobed margins, with numerous bifurcating parallel veins and no distinct midrib, leaflets not articulated; <b>basal offsets:</b> common.</p>
Chigua D.W/Stev.		2	Colombia	<p><b>Megasporophylls:</b> in spirally assembled stalked female cones; ovules: 2 (rarely 3), sessile; <b>microsporophylls:</b> spirally assembled into stalked male cones; <b>seeds:</b> with a red or pink fleshy outer sarcotesta; <b>stem:</b> subterranean, pachycaul &amp; globose; with few leaves; <b>leaves:</b> pinnate, spirally arranged, lower leaflets not reduced to spines; <b>leaflets:</b> articulated, simple, with dentate margins and numerous bifurcating parallel veins, and a distinct midrib.</p>

Table 1 contd.

Ceratozamia Brongn.	16	Mexico, Guatemala & Belize	<p><b>Megasporophylls:</b> in spirally assembled stalked female cones; <b>ovules:</b> 2 (rarely 3), sessile; <b>microsporophylls:</b> spirally assembled in stalked male cones; <b>seeds:</b> with a cream or white fleshy outer sarcotesta; <b>stem:</b> aerial or subterranean, pachycaul, cylindrical or globose; with few to many leaves; <b>leaves:</b> pinnate, spirally arranged, lower leaflets not reduced to spines; <b>leaflets:</b> articulated, simple, entire, with numerous bifurcating parallel veins and no distinct midrib; leaf bases mostly not persistent; new leaves emerging singly or in flushes; <b>basal offsets:</b> in some cases.</p> <p><b>Megasporophylls:</b> in spirally assembled stalked female cones; <b>ovules:</b> 2 (rarely 3), sessile; <b>microsporophylls:</b> spirally assembled in determinate, sessile male cones; <b>seeds:</b> with a red outer sarcotesta; <b>stem:</b> erect, aerial, &amp; cylindrical, with many leaves; new leaves emerging in flushes; <b>leaves:</b> pinnate, spirally arranged, lower leaflets not reduced to spines; <b>leaflets:</b> simple, with numerous parallel veins and no distinct midrib.</p>
Lepidozamia Regel	2	E Australia	

Table 1 contd.

<i>Macrozamia</i> Miq.	38	E Australia (34 species), Central Australia (1 species), SW Australia (3 species)	<b>Megasporophylls:</b> in spirally assembled stalked female cones; ovules: 2 (rarely 2), sessile; <b>microsporophylls:</b> spirally assembled in determinate, stalked male cones; <b>seeds:</b> with a red or less commonly yellow, orange or brown fleshy outer sarcotesta; <b>stem:</b> aerial or subterranean, pachycaul & cylindrical, with few to many leaves and persistent leaf bases; <b>leaves:</b> pinnate, spirally arranged, lower leaflets often reduced to spines; <b>leaflets:</b> simple or dichotomously divided, with numerous parallel veins and no distinct midrib.
<i>Zamia</i> L.	50	South, Central & North America	<b>Megasporophylls:</b> in spirally assembled stalked female cones; ovules: 2 (rarely 3), sessile; <b>microsporophylls:</b> spirally assembled in stalked or sessile male cones; <b>seeds:</b> with a red, orange, yellow or rarely white fleshy outer sarcotesta; <b>stem:</b> aerial or subterranean, pachycaul, cylindrical or globose, with few to many leaves; <b>leaves:</b> pinnate, spirally arranged, lower leaflets: not reduced to spines; dichotomous branching common in geophytic species; <b>basal offsets:</b> common.

Table 1 contd.

Table 1 contd.

Microcycas (Miq.) A. DC	1	Cuba	<p><b>Megasporophylls:</b> in spirally assembled stalked female cones; ovules: 2 (rarely 3), sessile; <b>microsporophylls:</b> spirally assembled in sessile male cones; <b>seeds:</b> with a red, fleshy outer sarcotesta; <b>stem:</b> aerial, pachycaul, &amp; cylindrical, with many leaves; <b>leaves:</b> pinnate, spirally arranged, lower leaflets not reduced to spines; petioles lacking prickles; <b>leaflets:</b> simple, entire, with numerous bifurcating parallel veins and no distinct midrib, leaflets articulated, dichotomous branching uncommon, <b>basal offsets:</b> rare.</p> <p><b>Megasporophylls:</b> in spirally assembled stalked ovoid female cones; ovules: 2 (rarely 3), sessile; <b>microsporophylls:</b> spirally assembled in stalked, ovoid male cones; <b>seeds:</b> with a dark red sarcotesta; <b>stem:</b> naked and often dichotomously branched &amp; subterranean; new leaves single; <b>leaves:</b> pinnate; <b>leaflets:</b> with a terminal leaflet, lower leaflets not reduced to spines; petioles lacking spines or prickles; flat, penniveined, with a large midrib and numerous sub-parallel, bifurcating lateral veins.</p>
Stangeriaceae	Stangeria T. Moore, Hook	1	S. Africa

Table 1 contd.





**Table 2.** Relationships among the 31 *rbcL* sequences of *Encephalartos*. The three parsimony informative positions were used to characterize chloroplast DNA types.

<i>cp</i> -DNA type	<i>rbcL</i> position 573	<i>rbcL</i> position 673	<i>rbcL</i> position 688	Taxon/specimen
1	A	A	G	<i>E. cupidus</i> , <i>E. cycadifolius</i> , <i>E. dyerianus</i> , <i>E. ghellinckii</i> , <i>E. gratus</i> , <i>E. hildebrandtii</i> <i>E. humilis</i> , <i>E. inopinus</i> , <i>E. kisanbo</i> , <i>E. laevifolius</i> , <i>E. lanatus</i> , <i>E. latifrons</i> , <i>E. lehmannii</i> , <i>E. longifolius</i> , <i>E. manikensis</i> , <i>E. munchii</i> , <i>E. princeps</i> , <i>E. villosus</i> , <i>E. whitelockii</i>
1	A	A	A	<i>E. aplanatus</i> , <i>E. cerinus</i> , <i>E. ferox</i> , <i>E. heenanii</i> , <i>E. ngoyanus</i> , <i>E. senticosus</i> , <i>E. umbeluziensis</i>
2	G	C	G	<i>E. natalensis</i> , <i>E. woodii</i>

relationships is composed of *E. aplanatus* and *E. villosus* 1, and defined by ISSR band 9. All other relationships found by ISSR support neither ITS 1&2 nor *rbcL* topologies [25].

The differences of the phylogenies of nuclear DNA, cpDNA and genomic fingerprinting (ISSR) suggest that hybridization and introgression played an important role in the phylogenetic history of *Encephalartos*. The differences in topology may be a consequence of the diverse behaviour of the three molecular markers to hybridization between species of *Encephalartos* and subsequent backcrossing. Even today, when the distribution of the species is strongly disjunct, several cases of natural hybrids were found [4, 28], and in phases of global cooling all the occurrences of *Encephalartos* may have been contracted and expanded repeatedly from eastern South Africa. The occurrence of hybrids from the three species *E. altensteinii*, *E. arenarius* and *E. lehmanii* [28] suggest that hybrids in *Encephalartos*, at least between several of the species, are fertile. The absence of hybridization barriers, together with

the small genetic distances of ITS 1&2 (maximally 3.7% p-distance), *rbcL* (maximally 0.3% p-distance) and allozyme markers [26, 27], suggest that the formation of the extant lineages of *Encephalartos* occurred recently rather than in Cretaceous times when the first fossils were assigned to this genus [13].

ITS 1&2 reveal that the genus *Encephalartos* displays its maximum genetic diversity (3.7% ITS 1&2 p-distance) in the mountainous regions of eastern South Africa, where the most basal ITS-group 1 as well as most taxa of ITS-groups 2 and 3, are located. This region, extending from the catchment area of the Limpopo to Port Elizabeth, is well-known as the Pleistocene refugium and diversification centre in several angiosperm groups [30]. Taking into account, the genetic diversification of *Encephalartos* in this region and the evidence from other plant genera, this area might also have been the centre of origin of the extant lineages of *Encephalartos* and refuge area of the ancestors in the Pliocene/Pleistocene global cooling period. Occurrence of all three ITS-groups in this region, together with the small genetic distances between the tropical African species and those from eastern South Africa, suggest that the spatial disjunction over large regions of Africa may have been caused by recent long-distance dispersal (LDD) rather than by shrinkage of a former wide distribution area into island-like occurrences and subsequent differentiation [10].

## **MOLECULAR CLOCK: AGE ESTIMATE OF CYCAD SPECIATION**

Since cycads represent an old plant family with a former Gondwana distribution, it is tempting to explain the vicariance of extant cycad genera on different continents by plate tectonics as nucleotide and amino acid sequences change with time [5, 17], the molecular clock approach offers a chance to discuss the evolutionary scenario of cycads.

For dating the divergence times, the approach of Savard et al. [18] was applied, using non-synonymous substitutions to calibrate the *rbcL* clock. Pairwise non-synonymous p-distances and divergence times were taken from Savard et al. [18] for calibration (taxon pairs: non-synonymous distance – divergence time): Liverworts-vascular plant split: 5.67% – 440 mya; fern-seed plant split: 4.67% - 395 mya; monocot-dicot split: 3.31% - 200 mya; divergence within Pinaceae: 0.71% - 140 mya.

For *rbcL*, rates were found to be homogeneous between gymnosperms, perennial angiosperms, fern and liverwort [18].

Using non-synonymous substitutions and four landmark events [18], a linear regression was calculated and used to date the divergence times within and between *Encephalartos*, *Lepidozamia*, *Macrozamia* and *Cycas* (Fig. 4) [24, 25]. Since cycads are gymnosperms with a long generation time, compared to that of the pine family (the latter factor is supposed to influence the substitution rate), the non-synonymous substitution rate variation between cycads and Pinaceae was calculated using the formula of Gaut et al. [5]. The distances within *Encephalartos* are unexpectedly small in *rbcL* (Table 3), as well as in the ITS region and between *Encephalartos*, *Lepidozamia* and *Macrozamia* (Table 3). The maximum

**Table 3.** Pairwise non-synonymous p-distances and calculated divergence times between Cycads and other vascular plants based on the *rbcL* gene. \* Divergence times taken from Savard et al. [18], used to calibrate the molecular clock (from [24]).

Taxon-pair	Genetic distances of non-synonymous bases ( )	Maximal divergence time (mya)
Liverworts-vascular plants*	4.44	440*
Ferns-seed plants*	3.40	395*
Monocots-Dicots*	3.81	200*
Diversification of Pinaceae*	1.21	140*
<i>Cycas</i> -other Cycads	1.48	132
Cycads except <i>Cycas</i>	1.05	94
Within <i>Cycas</i>	0.40	36
<i>Lepidozamia</i> - <i>Macrozamia</i> - <i>Encephalartos</i>	0.22	20
Within <i>Encephalartos</i>	0.11	9.8

non-synonymous distances and divergence times found were (average and standard deviations in brackets): Cycadaceae–Zamiaceae/Stangeriaceae-split: 1.19% ( $0.65 \pm 0.28$ ) – maximally 92.5 mya ( $50.2 \pm 21.7$ ); within Zamiaceae/Stangeriaceae: 0.95% ( $0.45 \pm 0.26$ ) – maximally 73.9 mya ( $35.2 \pm 20.5$ ); within *Cycas*: 0.12% ( $0.08 \pm 0.05$ ) – maximally 9.33 mya ( $6.2 \pm 4.4$ ); *Lepidozamia*-*Encephalartos*-split: 0.25% ( $0.16 \pm 0.06$ ) – maximally 19.4 mya ( $12.7 \pm 4.8$ ); within *Encephalartos*: 0.13% ( $0.08 \pm 0.06$ ) – maximally 10.1 mya ( $6.5 \pm 4.6$ ).

To confirm that the *rbcL* molecular clock is not irregularly slow in Cycads, the Relative-Rate Test choosing *Magnolia* as reference taxon was applied. Since the Relative-Rate Test could be sensitive to taxonomic sampling [16], four GenBank sequences of the Pinaceae were chosen to check for rate variation within the lineages leading to the four cycads *Encephalartos septentrionalis*, *Zamia spartea*, *Stangeria eriopus* and *Cycas revoluta*. The Relative-Rate Test revealed that the *rbcL* evolutionary rate is approximately 0.64 times slower in Cycads than in Pinaceae, which would not change the dimensions of divergence times and the main conclusion of our results. Due to the minor reduction of the substitution rate compared to the Pinaceae, it seems more likely to us that the extant species of cycads are the result of recent, rather than old, radiation.

Our molecular clock estimates agree with the fossil record showing that extant cycads evolved in the Tertiary era [7].

The African-Australian disjunction with low *rbcL*-divergence between the species of *Encephalartos*, *Lepidozamia* and *Macrozamia* (Figs. 4 & 5, Table 3) could be explained by long-distance dispersal in the Miocene, rather than by vicariance and continental drift. Another argument against a possible Gondwana origin is the observation that the Zamiaceae are absent from other Gondwanan crustal fragments, such as India, New Zealand, New Caledonia and South America.

The colonization of Madagascar by *Cycas thouarsii*, far from the distribution areas of the other *Cycas* species, seems to be even younger. The *rbcL* sequences of *Cycas thouarsii* and *Cycas rumphii* are identical, suggesting a recent dispersal from Indonesia to Madagascar. Since this cycad has a spongy endocarp, a transoceanic distribution appears plausible. Dispersal via the sea is also relevant for other members of *Cycas* subsection Rumphiae, since *Cycas* seeds remain viable even after long time in seawater. It is, therefore, noteworthy that the members of the subgroup Rumphiae occur both on the mainland of Southeast Asia and are widely distributed on islands of Indian and western Pacific oceans. An alternative idea for the settlement of Madagascar has been communicated to me by an ethnologist. Anthropologists suggest that the natives who colonized Madagascar came from Southeast Asia. In this region, Cycads are used for food. Therefore, it could be possible that the early settlers brought *C. thouarsii* directly to Madagascar as a potential food plant.

## Acknowledgement

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## Molecular Variability and Diversity of Mediterranean Pines: *Pinus halepensis* Mill. and *Pinus brutia* Ten.

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### ABSTRACT

Knowledge of the life history and ecological characteristics of woody plant species helps in predicting the level and distribution of genetic diversity within and among populations. The plant communities, which grow in a particular place, are influenced by their phytogeographical position, climatic factors, soil and human activities. The acquired changes are reflected in the genomic structure of forest trees during evolution. Genetic diversity in nature is the result of evolutionary processes, and investigation of molecular polymorphism is a quick way of understanding genetic changes in forest populations and the evolutionary development. Trees, which survived in new margins of environmental conditions, assist in identifying the forest boundaries and the evolutionary processes. Thus, in response to environmental changes, some of tree forest species may acquire new traits. These traits may allow many tree species to survive predicted global climatic changes while preserving much of their genetic diversity, advancing their evolutionary path in the new habitat.

**Key Words:** Genetic diversity, *P. halepensis*, *P. brutia*, pine evolution, ecological adaptation, population genetics

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## INTRODUCTION

Knowledge of the life history and ecological characteristics of woody plant species assists in prediction of the level and distribution of genetic diversity within and among populations. Generalizations developed from such analyses can be used to develop sampling strategies for the preservation of genetic diversity.

Lately, evolutionary history of plants has been studied by investigating mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA). The main advantage of these genomes is that they allow separate research for maternal and paternal lines, as mtDNA is transferred to the offspring usually only from the mother. This analysis helps in the revelation of the evolutionary past of plants. Undoubtedly, the mtDNA and cpDNA represent only a small part of the plant genome, and these parts differently reflect migration and selection events. However, minor details might be of great importance for revealing the complete picture. Accordingly, research of these genomes, together with the nuclear genome, may reconstruct the objective picture of the evolutionary paths for plants.

The first gymnosperms arose in the Middle Devonian (~365 million years ago). Palaeobotanical discoveries have shown that ancestors of *Pinaceae* family evolved by the mid-Jurassic period (~160 million years ago). More than half the species in the *Pinaceae* are included in the genus *Pinus* (100 species) [73] representing 20% of all gymnosperms. The genus *Pinus* is divided into two main subgenera, *Pinus* (hard pines) and *Strobus* (soft pines), diversified by the end of the Cretaceous (66 mya) [54, 76]. Several sections (e.g. *Strobus* and *Pinus*) and further subsections (e.g. *Sylvestres*, *Attenuatae* and *Strobi*) have evolved since the diversification of these two subgenera [44, 73].

The impact of the Eocene had the effect of dissecting the genus and concentrating pines into widely disconnected regions. During the Pleistocene (1.7 - 0.01 mya), pine populations shifted first south, then north. The climatic fluctuation during the Pleistocene may have played an important role in the speciation and preservation of distinctive genotypes [76]. For the last 10,000 years after the last glacial period, the current distribution of pines has been shaped. Pines are the most

widespread tree genus in the world and are of ecological and economic significance. The natural distribution of pines encompasses the area from North America and Eurasia south to subtropical and tropical regions of Central America and Asia [54, 73].

## GENOME ORGANIZATION OF CONIFER

Pines form an important group of evergreen plant species, which are extensively distributed worldwide. This group is characterized by high genetic variability in comparison to other higher plants [31]. Although conifers are the largest and most diverse group of living gymnosperms, the relationship between families and species is not clear [91]. An important characteristic of the conifer genome is its large size compared with other plant species. Among eukaryotic organisms genome size varies several hundredfold, thus among angiosperms the DNA content variation is from  $2C = 0.1$  to 254 pg. [4]. The variation of the genome size between 20 *Pinus* species collected in North America, Europe, and Eurasia was 1.73-fold [67] and only in 18 North American *Pinus* species alone the variation was 1.5 fold [94]. In spite of the large size of the DNA content, the evolution of the conifer chromosome appears to be quite conservative. The number of chromosomes among conifers is not highly varied and is normally equal to 11 or 12. The number of genes, which cover genomic traits, their spatial organization and degree of variability among different trees, populations, provenances and species, remains largely unknown.

The size of the pine genome (20,000-30,000 million nucleotide base pairs [bp], for example, is 6 to 8 times larger than the human genome (3,400 million bp), and 150 to 200 times larger than the genome of model plant species, *Arabidopsis thaliana* (125 million bp). Even a relatively small physical size of the *Populus* genome (500 million bp), which is 40 times smaller than the best-studied conifer, *Pinus taeda*, and, therefore, can be a good forest tree model species, is still about 4 times as large as that of *Arabidopsis* (although similar to rice and 6 times smaller than maize. In the nucleus of the conifers, DNA content is also the largest among higher plants. In pine chromosome, the DNA content is approximately 3.7 times larger than in maize, 5.2 times than in lettuce, 15 times than in tomato, and 110 times than in *Arabidopsis* [60]. Although the size of the conifer genome in terms of DNA content appears very large compared with the size of other plant genomes, in

terms of map units it is not much greater than in other crops. Neale and Williams [60] estimated the pine genome to be approximately 2,500 centimorgans (cM), which is only two times larger than tomato, corn or lettuce. Since the main limitation of the pine genome measurement with allozyme technique is a small number of markers, it is possible to elevate the resolution in genome evolution research by applying modern technologies. Studies of conifers based on allozyme markers have mapped only about 10% of the genome (226.4 cM) [13]. With the help of random amplified polymorphic DNA (RAPD) for mapping of *Pinus elliotii*, it was shown that 64-75% of the total genome size is approximately 2,160 cM [63]. In *Pinus taeda* 191 RAPDs were mapped to 12 linkage groups with a distance of 1,687 cM, and in *Pinus silvestris* 282 RAPDs formed 14 linkage groups with a total distance 2,638 cM [104]. For *Pinus brutia* the total map covers a distance of 662.8 cM [34]. Sewell et al. [86] estimated the genome size of loblolly pine (*Pinus taeda*) as 1,227 cM., whereas a constructed genetic map of loblolly pine from all available genotypic data for comparative analyses among pine genomes (i.e. 12 linkage groups) consisting of 155 RFLPs, 75 ESTPs, and 5 isozyme loci is similar to the conservative estimation from Sewell et al. [86] - 1165 cM [8].

The chloroplast genome structure has been studied in a variety of plants. In many species it generally consists of homogeneous circular double-stranded DNA molecules with a size of 120-160 kb. An outstanding feature of chloroplast DNAs (cpDNAs) found in most plants is the presence of a large inverted repeat (IR) of 6-76 kb [71]. The complete nucleotide sequence of the black pine (*Pinus thunbergii*) chloroplast genome is 119,707 bp [96]. The chloroplast genome is highly conservative and has a much lower mutation rate than that of plants with nuclear genomes. However, the level of polymorphism depends on the taxa or species. Some regions in the chloroplast genome are highly conserved and display an absence of polymorphism. The other regions are more variable. In Angiosperms a small variation between populations was discovered, and no intra-population variations were found, possibly due to the maternal mode of inheritance [72]. In view of the importance of conifers among particular forest trees, their evolutionary distance and divergence may be determined from chloroplast-specific polymorphic assay.

In all genomes only a very small amount of DNA apparently has coding functions and most of the pine genome is made of repetitive

sequences. Changes in the genome size possibly involve a variation in the amount of repeated DNA sequences [6, 22, 24, 97].

There are two hypotheses, which may explain why DNA is larger in conifers than in other plants. One hypothesis is that conifers may have a higher content of repetitive DNA than other plants [19, 43]. The repetitive DNA fraction in conifers is approximately 75%. The other 25% of DNA is equivalent to DNA of plants with much smaller genomes.

An alternative hypothesis is that there must also be an extra amount of the single-copy DNA [43]. This hypothesis is supported by the fact that rRNA genes in conifers are more repetitive than in other plants. Flavell et al. [23] showed in *Triticum dicoccoides*, that rDNA diversities are correlated with climatic variables in Israel. Significant correlations between the *Pinus* genome size and climatic factors (temperature and precipitation) were observed [66, 94, 95]. According to these correlations, it was suggested that the large genome size and its variation in *Pinus* was a response to the habitat conditions of these species. Thus, the model for genome evolution presumes that the plant genome is flexible and has repeatedly undergone amplification and deletion of DNA sequences over evolutionary time. However, the functions of repetitive DNA and its role in genome organization are not clear.

## MOLECULAR POLYMORPHISM

Genetic diversity may be estimated by using measurable traits, but they are unable to provide information about which particular genes or how many of them are involved in adaptation. Another, and generally complementary, approach for estimating adaptive genetic diversity is by measuring genetic variation using molecular genetic markers. Numerous molecular markers were used in forest trees to understand their genetic structure and evolution, as well as to find changes for evaluation of large numbers of adaptive genes and genetic variations. Pines are diploid organisms with a constant number of haploid chromosomes equal to 11 or 12. That may be the reason why small karyotype differentiation was shown within the genus by cytological studies [70, 78]. Accordingly, the use of genetic markers for the detection of adaptive traits - which are very complex and are controlled by many genes, each with relatively small effect - is acceptable and may be very successful.

The first widely used method of polymorphism detection in pines was allozymes. In most studies allozyme markers have been used as a basic tool for providing genetic information [38]. Of approximately 50 species studied with these markers, at least one estimate of expected heterozygosity was obtained. These markers have provided most of the data on the genetic structure of plants in general, and trees in particular. Unfortunately, the sensitivity of allozymes is about one-fourth of the total number of bases sampled. The benefit is that these markers are codominant. Studies of biochemical markers have shown that forest trees, particularly conifers, are among the most variable organisms known. Therefore, a great interest of ecologists and foresters is associated with studying native variability by molecular markers and understanding mechanisms of genetic differentiation and evolution of genomes.

In angiosperms, the mitochondrial and chloroplast genomes are generally inherited maternally. In conifers the situation is different. According to the genus and the species, the organelle genome can be inherited maternally, paternally or both ways. The plant mitochondrial genome is highly variable in structure but shows a very slow rate of nucleotide substitution, despite its large size – due to the presence of introns, intergenic sequences, duplicate sequences and sequences of plastid and nuclear origin in higher plant mtDNA. mtDNA is as conservative as the chloroplast genome, and as a result only a few mtDNA markers are available and this slows down the evolutionary studies in conifers.

The molecular biological advances are focused on DNA markers, such as random amplified polymorphic DNA (RAPD) [34, 63, 104] and simple sequence repeat (SSR) markers [91, 92]. Molecular markers, such as restriction fragment length polymorphisms (RFLP), are more powerful in revealing genetic polymorphism; however they are technically more demanding and expensive.

RAPD markers are a type of genetic markers which are based on the polymerase chain reaction (PCR) [102]. The markers are inherited in a Mendelian manner and can be successfully generated for any species without prior DNA sequence information. The ability of RAPD primers to produce multiple bands using a single primer means that a relatively small number of primers can be used to generate a very large number of fragments. These fragments are usually generated from different regions

of the genome, hence they appear as multiple loci. RAPD markers are especially well suited for indicating genotypic distinctions at the clonal level, since an increasing proportion of the total genome can be sampled by the use of additional primers until genotypic differences are discovered.

The single stranded repeats (SSRs) are DNA sequences that are repeated in a tandem number of times. The larger repeats (up to 5 Mb) are called satellites. Intermediate repeats (the repeated units that are bigger than 10 bp and form blocks of 0.5 to 30 kb) are called minisatellites. Microsatellites have repeated units of 1-8 bp and form structures that have 20 to 100 bp. Microsatellites are co-dominantly inheritable, which allow for the discrimination of homo- and heterozygotic states in diploid organisms during population analysis [45]. The nuclear and chloroplast microsatellites were identified for population analysis in *Pinus contorta* [32], *Pinus radiata* [21, 87], *Pinus strobus* [20], *Pinus silvestris* [41, 88], *Pinus brutia* and *Pinus halepensis* [9, 35].

Lately, the measurement of genetic diversity using single nucleotide polymorphisms - SNPs - has been acquiring much popularity. According to the accepted definition [7], SNP is a single-nucleotide position in genomic DNA which has various variants of sequences (alleles) in some populations, while the rare allele's frequency is at least 1%. Sometimes SNPs with frequency of rare allele higher than 20% are defined as "abundant SNPs". However, quite often, all the small changes in the genome sequences, which were detected during SNP screening, are placed in same databases. Theoretically, the presence of two-, three- and four-allelic polymorphic forms is possible. However, in practice, presence of even three-allele SNPs is extremely rare in genome (less than 0.1% of the whole individuals SNP). Four different types of two-allele SNPs can be found. Practical interest in SNPs had increased during realization of projects, in which the full nucleotide sequences of some organisms were researched. A huge quantity of SNPs in the human genome (about 3-10 million of SNP) allows for a selection of about 100,000 SNP markers [48]. Thus, in every known or assumed gene, there are at least two appropriate markers.

The above methods of molecular polymorphism investigation are not exclusive, because the design and development of new methods in this field are continuing.

## DIFFERENCES IN *P. halepensis* AND *P. brutia* AS EXAMPLES IN THE EVOLUTIONARY HISTORY OF MEDITERRANEAN PINES

Until 1952, *P. halepensis* and *P. brutia* were thought to be two varieties of a single species (i.e. *P. halepensis* var. *halepensis*, and *P. halepensis* var. *brutia*). By analyzing morphological parameters in seedlings of *P. halepensis*, *P. brutia*, *P. eldarica*, *P. stankeweczii* and *P. pithyusa*, Debazae et al. [18] concluded that *P. halepensis* and *P. brutia* are separate species, with three relict pine taxa, namely *eldarica*, *pithyusa* and *stankeweczii*, being subspecies of *P. brutia*. There are significant differences between *P. brutia* and *P. halepensis* trees in their morphological characteristics, i.e. bark color, needle length, width and cross-section structure, cone structure and 1,000-seed weight [84, 93]. By using data obtained from the physical and chemical analysis of the resin turpentine as genetic markers, Mirov et al. [53] concluded that *P. halepensis* and *P. brutia* are two distinct species. According to Conkle et al. [14] allozymes indicate a highly significant divergence between *P. brutia* Ten. and *P. halepensis* Mill.

A number of differences were also revealed in phenological trials (i.e. periodical phenomena of flowering and vegetative growth) of *P. halepensis* and related species, *P. brutia* and *P. eldarica* [100]. In the phenological stages, which were observed during an annual cycle, changes in the development of reproductive and vegetative organs were found. The phenological phenomena, being subject to natural selection pressures such as the timing of frosts and droughts and insect behavior, reflect an adaptive response to environmental heterogeneity [10]. A different approach to distinguishing between these two species was based on karyotype analysis. According to Saylor [79] and Kammacher [33] significant differences occur mainly in the 11th and 12th chromosomes.

The relationships between *P. halepensis* and other Mediterranean pines are still not evident, the origin and the migration pathways and the past areas of distribution are unclear. On the basis of palaeobotanical data, Nahal [58] concluded that in the Tertiary *P. halepensis* was growing in what is now known as the Baltic Sea area. Panetsos [68] postulated the existence of two centers of distribution of *P. halepensis* and *P. brutia*: the former in northwestern Europe, and the latter in Eastern Europe. Changes in the climate forced a southward shift of these two species and, according to their physiological-ecological characteristics, *P. halepensis* established itself mainly in the western Mediterranean, and *P. brutia* in



the eastern Mediterranean region. The eastern group is represented by the Israeli composite sample, and probably includes the native Jordanian and Lebanese-Syrian provenances. There are distinct additional alleles, which are widespread in Israel and rarely or even not detected in other *P. halepensis* or *P. brutia* populations [80]. Pines of the *P. brutia* group are polymorphic and are presumed to have more variation than *P. halepensis*. The allozyme similarities of *Pinus eldarica* (Medw.) to the easternmost *P. brutia* subspecies *brutia* (Nahal) population and its reduced diversity provide evidence of its derivation from subspecies *brutia* [14].

The evolutionary history of *P. halepensis*, reconstructed from allozyme evidence, indicated that the center of origin included the regions bordering the Black Sea and easternmost Anatolia, with eastward extensions into the lands between the Black and Caspian Seas. Early populations may have been more widespread, larger in size, and more closely adjacent. *P. brutia* is a characteristic species of the eastern Mediterranean, whereas *P. halepensis* generally occupies the western and middle Mediterranean - except for local occurrence in the southern parts of the eastern basin. *Brutia*, the modern subspecies around the Aegean and Mediterranean Seas (western population), is a widespread subspecies that maintains significant levels of allozyme variation throughout its geographic distribution. The eastern population of *brutia* subspecies is now geographically isolated from the main distribution. Several of the eastern populations resemble *stankeweczii* (Suk.), *pithyusa* (Steven), and *eldarica* (Medw.) subspecies in possessing rare alleles and in having allele frequencies, which distinguish them from western populations of *brutia* subspecies. The morphological differentiation among subspecies *brutia* was sufficient to assign species status to them, but enzyme allele frequencies of these subspecies closely resemble those of subspecies *brutia*. Analysis of chloroplast microsatellites in natural populations of *P. halepensis* and *P. brutia* identified "species-specific" haplotypes in the two species [9]. At species level, two species are clearly divided into two main clusters with further subdivision, showing genetic divergence among populations of *P. halepensis* and *P. brutia*.

The research of sequence divergence of chloroplast *rbcl*. *MatK* *trnV* intron and *rpl120-rps18* spacer regions among 32 *Pinus* species and members of six other *Pinacea* genera showed that within the Mediterranean pine clade, *P. halepensis* and *P. brutia* formed a highly supported group of Wang *et al.* [98]. A clear resemblance in their seed protein profiles [85] and allozyme pattern [14], and their ability to



hybridize in nature [69] - indicate a close relationship between the two. *Pinus brutia* is even described as a variety of *P. halepensis* by some authors [84, 100]. Allozyme [14] and morphology [25] studies have suggested that *P. halepensis* is derived from a *P. brutia*-like ancestor and that *P. brutia* has retained greater ancestral variation, showing affinities not only to *P. halepensis*, but also to other Mediterranean pines, e.g. *P. pinaster* and *P. canariensis* [25]. *Pinus pinaster*, *P. pinea* and *P. canariensis* formed one group, albeit with weak (<50%) bootstrap support. Many authors consider *Pinus pinea* as an enigmatic and isolated species [36, 53]. Traditionally, *P. pinea* is placed in a monotypic subsection *Pinea* [50]. However, Wang et al. [98] did not observe such a distinct separation of *P. pinea* from other Mediterranean pines. Klaus [36] noted that *P. pinea*, *P. pinaster* and *P. canariensis* share many cone and vegetative characters. Frankis [25] combined *P. pinaster*, *P. canariensis*, *P. halepensis* and *P. brutia* into one subsection, *Pinaster*, but both authors still placed *P. pinea* in a separate subsection. The results [98] lend additional support to the grouping of these species into one subsection, *Pinaster*, as suggested by Frankis [25], but indicate that *P. pinea* may also belong to this subsection.

## GENETIC ALTERATIONS AS ADAPTATION TO ENVIRONMENTAL CHANGES

Genetic diversity is the basis for the ability of organisms to adapt to changes in their environment through natural selection. During their evolutionary and ecological histories, forest tree species have experienced numerous environmental changes. Shifts in climate could take place for a thousand years (glacial epochs), or last for the lifetime of an individual—a couple of decades. Environmental changes could be smooth, or it could be rapid climatic shifts taking place for only a few years. For the past few years much attention has been focused on the research of genetical diversity of tree species due to the environmental changes related to human activity [2, 16]. Simultaneously, both the degree and the rate of climatic changes are of relation to the future of tree species. Firstly, the rate of environmental changes could be too rapid for trees with their long generation time to adapt to it [17]. Secondly, there are many concerns that the extension of environmental changes could be much bigger than the genetic adaptation abilities of the plants [16]. Thirdly- and finally- even if trees could have proper genetic diversity for an adequate response to the rate and strength of climatic changes, it is possible that they might

not be capable of spreading out into newly available habitats quickly enough to match the rate of environmental change [12].

Aleppo pine (*Pinus halepensis* Mill) is due to one of the most widespread forest tree species in the Mediterranean its optimum adaptation to the microclimates of the area, i.e. its high tolerance to drought and adverse ecological conditions [59, 99]. This species has some features of its reproductive cycle and the production of serotinous cones, which are important for natural distribution and regeneration following fire passage [46, 77]. Its area of growth stretches from Morocco (longitude about 9°W) to Jordan (longitude 36°E) and widens from France (latitude about 45°N) to Israel (31°30'N) [15, 84]. Within the Mediterranean Basin alone, natural forests and forest plantation of *P. halepensis* (Mill) cover over 3.5 millions ha. [29, 58, 90]. However, the main area of *P. halepensis* distribution is southern Europe and North Africa.

Natural populations of *Pinus halepensis* have high survival capacity and occupy mountain sites as well as low-altitude sites, reaching elevations up to 2,100 m [68]. Individual populations grow in deserts with a minimal amount of annual rainfall. However, natural populations showed a high degree of similarity based on results of researching the characteristics of seeds, seedlings, needles and cones [51, 52]. Trees are subjected to more variation due to drought than any other plants, and the effect is often more devastating [28]. Tree diameter growth [42], height growth [106], and the number of shoot flushes produced in a growing season [105] can be substantially reduced by drought. Drought stress is a common cause of seedling mortality in both naturally regenerated and planted stands of *Pinus* species. Williston [103] noted that over a 16-year period, 57% of the first year mortality in pine plantations was due to drought stress. In established stands, drought stress accounts for 80% of the variation in annual ring width of conifers in humid temperate climates [107], and up to 90% in semi-arid regions [26]. The ability to survive drought stress has been shown to be variable among geographic seed sources of *Pinus* [101]. Various authors have suggested adaptation to local environmental conditions as an explanation for differences in allele frequencies in forest tree species covering large geographic distances. In other words, genetic differentiation in species is suggested to be a basis of micro geographic adaptation. The survival and adaptive possibility of *Pinus halepensis* in Israel supposedly led to

formation of new provenances adapted to local environmental conditions [31, 39, 40, 65]. Unfortunately, the short distance between separate provenances of *Pinus halepensis* may lead to an appearance of mixed stands due to “introversive hybridization”. The creation of intermediate morphologic forms is an indirect evidence of natural hybridization. Also, an indirect evidence of natural hybridization in populations of *P. halepensis* and *P. brutia* is based on morphological traits [68], allozymes [37] and microsatellites [9].

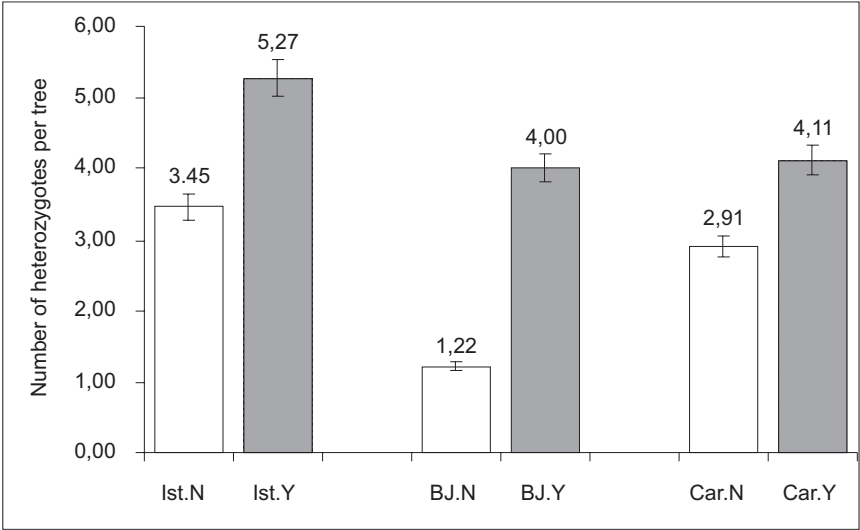
Within the Mediterranean Basin *Pinus halepensis* grows under various ecological conditions, which are the result of topography, climate and human activity. These conditions have always played a significant role in moving the geographic borders of this species. Therefore, there is a need to be very cautious in the interpretation of genetic diversity data in the geographical context.

## **ENVIRONMENTAL EFFECTS ON ADAPTIVE GENETIC VARIATION IN *PINUS HALEPENSIS***

Genetic diversity in nature is the result of evolutionary processes, and it is apparent within species at different levels in both the enzymes and the DNA [64, 65]. Nevo, in his “Evolution Canyon” model, showed correlations between environmental conditions and diversity of plant species. This parallelism between diversity and microhabitats suggests that genetic and physiological diversity should be represented as arising from a complex of adaptive factors, related to environmental heterogeneity. Natural climatic selection and stress appear to be the major differentiating factors. Apparently, the proportion of heterozygotes in a population is related to its adaptive effectiveness; accordingly, stressful conditions may change the adaptive capability of plant populations [31, 49]. Furnier and Adams [27] found correlation between allele frequencies and adaptation to ultramafic soils, and Guries and Ledig [30] established correlation between allele frequencies and climatic variables, such as winter temperature. In the Swiss sub-alpine stands of *Picea abies* and *Fagus sylvatica*, Müller-Starck [57] found relatively large intra-population and average inter-population genetic variation in comparison with reference populations in Europe. According to Müller-Stark [56, 57] and Bergmann and Ruetz [5] levels of genetic diversity in stressed plantations of forest tree species are similar or higher than those in favorable natural populations. Similarly, the numbers of heterozygous

phenotypes present in natural populations were found to be considerably lower than those in the offspring stressed populations in Israel [39, 40]. Climatic natural selection through water deficit and existence of significant selective factors in populations of *P. halepensis* in Spain were found when phenotypic traits and molecular markers were compared [1]. Heterozygous individuals are believed to be more stable than their homozygous counterparts because of some inherently superior biochemical efficiency possessed by heterozygotes [47].

Allozymes and RAPD markers were used to analyze the genetic variability among natural *Pinus halepensis* (Mill.) populations, and between their offspring populations, and to relate the intra-specific variability to the ecological differences among the sites. Both methods showed an increase of heterozygosity in populations growing in new environments. Most of the trees in provenances that survived under the stressful ecological conditions (an average of 44% of the number planted) were heterozygous (Fig. 1). Also, the fixation indices were negative at most of the enzyme loci, i.e. they indicated an excess of heterozygotes in



**Fig. 1.** Alteration of heterozygote number in main provenances of *P. halepensis*.

N-number of heterozygote in natural populations

Y-number of heterozygote in offspring populations in the stressful site

**Abbreviations and population names**

BJ - Bet J'ann –provenance (Israel), Car - Carmel provenance (Israel), Ist – provenance Istiaia (Greece)

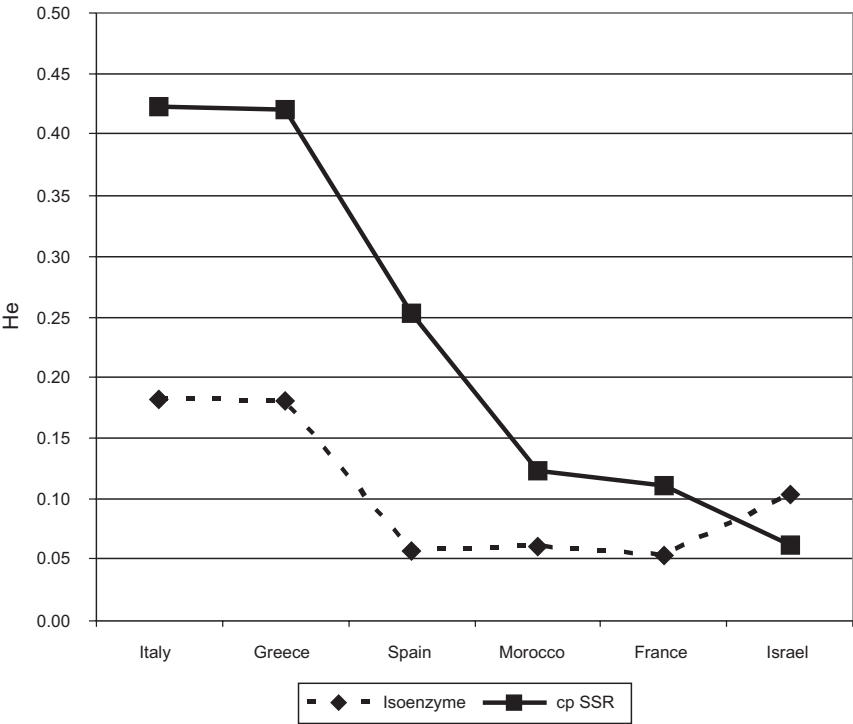
the F1 offspring populations. These results gave rise to the hypothesis that as a population adapts to new climatic conditions, an increase of genetic variability may be expected and the number of heterozygotes would correlate with environmental features. We consider that the correlation between gene diversity and survival rates, with negative correlation coefficients, suggests a model of heterozygosity in which heterozygous individuals are developmentally more stable than their homozygous counterparts [47, 89]. All these indicate that at least some of the inferred changes may be reflected in selective responses to environmental conditions that are also reflected in the low survival rate 15 years after planting.

Further natural selection should promote divergence within provenances at a micro-site; therefore, theoretically it seems reasonable to suppose that micro-site ecological-genetic differences can promote a tendency towards site-specific differentiation for stress-resistance traits. It is obvious that population size, history, and past and present genetic flow values are important in the determination of genetic heterogeneity and structure within and between plant populations. Nevertheless, adaptive selection is receiving increasing attention: non-selective processes, which cause loss of allele heterozygosity, probably influence all components of population adaptation.

## Diversity Between Circum-Mediterranean

By analyzing levels of within-population genetic diversity in natural populations of *P. halepensis*, published by Korol et al. [40], we have compared our results with the results of the distribution of genetic variability in the same populations measured by nine cpSSR [9] Fig. 2. In all probability, the correlation ( $r = 0.804$ ,  $P = 0.046$ ) between data obtained by different methods could represent an indirect confirmation of our supposition on the evolutionary history of this species in the Mediterranean Basin.

The analysis of the population diversity in Mediterranean *Pinus halepensis* revealed two main geographic aggregations that were very similar to the groups published in the earlier study by Schiller et al. [81] and Bucci et al. [9]. We have compared different types of the dendrograms constructed by methods of the nearest neighbor and UPGMA, on the basis of Nei's genetic distances [61, 62], Cavalli-Sforza's chord measure [11] and Reynolds, Weir, and Cockerham's [75] genetic



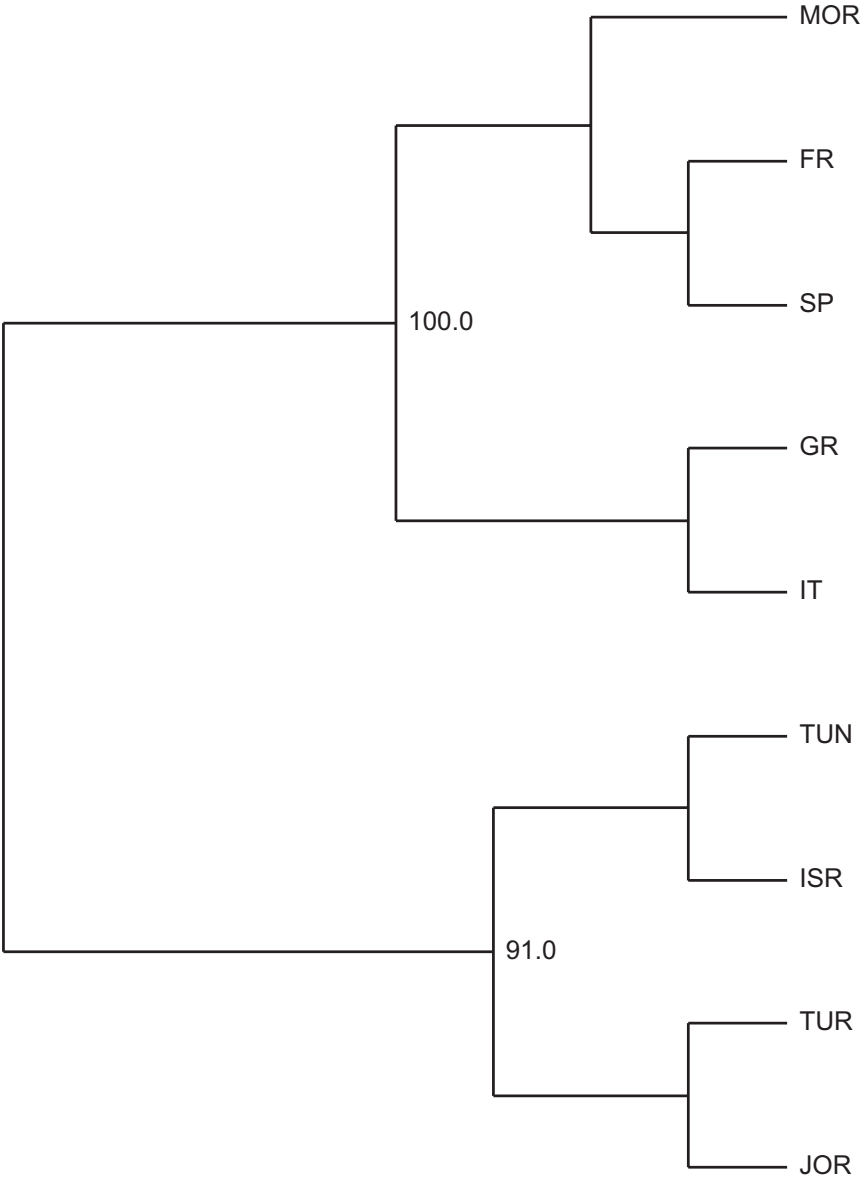
**Fig. 2.** Levels of within-population genetic diversity ( $H_e$ ) in natural populations of *Pinus halepensis* measured by two types of molecular markers.

distances, and have found that as a rule, populations of the same region are grouped together, regardless of the method of genetic distance measure used.

In Fig. 3 a dendrogram of *Pinus halepensis* is shown, which is created on the basis of Cavalli-Sforza's chord distance. We chose Cavalli-Sforza's chord distances, whose main advantage compared to other genetic distances is that its estimations do not depend on variations of population sizes during the time pace, and are very sensitive even to weak genetic drift in population. This is a very important advantage, for populations always vary in size.

The results of the analysis showed that all natural Aleppo pine populations in eastern Mediterranean, i.e. Turkey, Lebanon, Syria, Israel and Jordan, belong to the same group.

The eastern Mediterranean group differs from the western Mediterranean group mainly by the frequencies in the Cat2 and the Aap



**Fig. 3.** UPGMA dendrogram of *Pinus halepensis* populations from different regions of Mediterranean Basin, created on the basis of Cavalli-Sforza's chord distance. Numbers on branches leading to the main clusters are bootstrap values in percentage (of 100 replicates).

**Abbreviations and population names**

MOR – Morocco, FR – France, SP – Spain, GR – Greece, IT – Italy, TUN – Tunisia, ISR – Israel, TUR – Turkey, JOR – Jordan

loci. This divergence was also supported by the results of research by chloroplast microsatellites [9]. Similarity of allele frequencies in AAP and Cat2 loci points to a connection between Tunisian populations and eastern Mediterranean group, which strengthens former conclusions about the migration path of the Aleppo pine into the African continent. Native populations of *P. halepensis* from Tunisia have differences in genetic variability from the other populations forming a particular cluster in the dendrogram. A split among the Tunisian populations was also evident from the results presented in previous studies by Schiller and Grunwald [82] and Baradat et al. [3] using resin monoterpene composition as a genetic marker.

Western-Mediterranean group contains two subgroups: stands from Morocco were very similar to the southern French and Spanish stands on the one hand, and to the group comprising Greek and Italian stands of *P. halepensis* on the other hand. According to Schiller and Mendel [83], the Albanian, Greek and Italian peninsula Aleppo pine populations were considered as the eastern-European subgroup of the western-Mediterranean group, including an introgression from *Pinus brutia* [81]. Partially, evidence may be found in the work of Bucci et al. [9], where a possibility of hybridization between halepensis-complex pine species was proved by using microsatellites, although the authors themselves were supporting the hypothesis of Nahal [58], that genetic similarity of Greek and southern Italian populations is based on the migration of a limited number of individuals (founder effect) and regulation of population size by fire (bottlenecks).

Genetic similarity between Greek and Italian populations, having the highest genetic diversity in the whole range of this species may be represented as an importation of individuals by humans [74]. Schiller and Mendel [83] suggested that Aleppo pine in the Balkan Peninsula is "a direct descendant of Tertiary Aleppo pine in central Europe, which migrated into the Balkan Peninsula due to climate changes, keeping its relative high heterozygosity", which might be more believable. Such a hypothesis is supported by the findings of Morgante et al. [55], who categorically rejected the idea of introgression because of their interpretation of chloroplast microsatellites analysis results.

## CONCLUSION

Tree species have passed through large-scale global environmental changes many times during their evolutionary history. It is most likely



that although these changes occurred very quickly, many of the tree populations have survived. There are at least two main differences between the conditions today and during these historical events. Firstly, we currently experience quick global climatic changes during a period when many landscapes are very fragmented. As a result, secure sites for colonization may be so dispersed, that species might be unable to extend their dispersion in response to global ecological changes.

Secondly, however major areas of agricultural grounds were colonized by trees, following the abandonment of agriculture, in a very fast tempo. Also, it is likely that the ranges of commercially valuable species will be extended artificially into previously unoccupied areas. The establishment of plantations may expedite the colonization of adjacent habitats by the cultivated species and may also provide a suitably large target for non-commercial tree species to become established.

Moreover, as a response to environmental changes, some of tree forest species may acquire new traits. These traits may allow many tree species to survive predicted global climatic changes while preserving much of their genetic diversity and advancing their evolutionary path in the new habitat.

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# Survey of Genetic Diversity and Phylogenetic Relationships in Tunisian Date-palms (*Phoenix dactylifera* L.) by Molecular Methods

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## ABSTRACT

This study portrays the investigation of the genetic diversity and the phylogenetic relationships in Tunisian date-palms *Phoenix dactylifera* with the help of molecular markers (RAPDs, ISSRs and SSRs). The data provide evidence of a high genetic diversity in the local germplasm and prove the reliability of the designed methods as attractive approaches to examine the relationships in this crop. Compared to the RAPD, the ISSR procedure seems to be more useful in assessing the molecular polymorphism of this crop. As a result, the genetic diversity is typically continuous and the ecotypes studied are clustered independently from their geographic origin and from the sex of trees. Thus, it

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is assumed that a common genetic basis characterizes the ecotypes of the date palm despite their morphological distinctiveness. Moreover, the data are in agreement with the unique Mesopotamian origin of this crop.

**Key Words:** *Phoenix dactylifera*, molecular markers, polymorphism, phylogeny

## INTRODUCTION

The date-palm (*Phoenix dactylifera* L.) ( $2n = 36$ ), a dioecious perennial monocotyledon, is the most important fruit crop growing in arid and semi-arid areas of North Africa and several tropical countries. This is one of the oldest fruit crops known for its Mesopotamian origin in the fertile crescent [46, 83] and constitutes the major factor of oases' environmental and economic stability. The date-palm is cultivated either for food or for many other commercial purposes, and it maintains appropriate habitat for animals, mankind, subjacent vegetables, and other fruit crops (pepper, saffron, melon, fig, apricot, etc.) and constitutes the main financial resources of oasians. About 10% of Tunisians are dependent on its culture [21, 34]. Utilization of the palm consisted of locally adapted ecotypes; female trees, artificially pollinated and selected according to agronomic criteria, mainly the productivity and the date quality. These cultivars or varieties are clonally reproduced via offshoots [45, 50]. Recent investigations have proved that Tunisian date-palm germplasm is characterized by a large diversity since more than 250 cultivars have been identified [59, 60]. Unfortunately, this phylogenetic patrimony is seriously menaced either by a severe genetic erosion due to the predominance of the elite cultivar "Deglet Nour", or by plagues locally called the "brittle leaves", a disease of unknown origin, and the "bayoud disease", a fusariosis due to the fungus *Fusarium oxysporum* f. sp. *albedinis* [20, 80]. Hence, it has been imperative to elaborate a strategy aimed at the evaluation of the genetic diversity and the preservation of this important germplasm. For this purpose, many studies have been designed and have described the use of either morphological traits or isozyme makers to identify the Tunisian date-palm varieties [12, 51, 52, 58, 59]. However, these studies are less rewarding due to the limitation in the number of morphological markers that are highly sensitive to the environmental variations and despite their usefulness, the generation of isozyme markers is time consuming, their numbers are limited and their expression is often restricted to specific development states or tissues.

Therefore, to overcome these difficulties, DNA based techniques have been developed and have proved effective to assess genetic polymorphisms. It should be stressed that little is known about the date-palm's genetics and genome organization [16, 17]. These authors have proved that data based on the Restriction Fragment Length Polymorphisms (RFLPs) are suitable in the characterization of date-palm genotypes. Thus, a relatively important genetic variability among cultivars has been evidenced and the reliability of several parameters as efficient criteria to discriminate the cultivars studied has been also suggested. Thus, we became interested in the development of other molecular methods in order to characterize date-palm genotypes and to have a deeper insight of the genetic diversity in the Tunisian germplasm.

A large panel of available DNA based methods has been designed to examine the genetic organization in higher plants. Among these techniques, the Random Amplified Polymorphic DNA (RAPD) [82], the Inter Simple Sequence Repeats (ISSR) [32] and the Simple Sequence Repeats (SSR) methods are the most appropriate and widely used either in higher plants [4, 13, 75, 78] or animals [26, 27, 41, 57]. In fact, the RAPD and ISSR, a based DNA Polymerase Chain Reaction (PCR) technologies [65] are of several benefits over the other techniques since: firstly, little amounts of either plant material or DNA are required to perform DNA variation analyses; secondly, these methods are known either as informative about the molecular diversity or suitable in the discrimination of closely related genotype variants and the establishment of their DNA fingerprinting [2, 23, 33, 35, 85]; and thirdly, they have enabled the detection of polymorphisms without any previous knowledge of any crop DNA sequence. Moreover, RAPD and ISSR provide a nearly unlimited potential of markers to reveal differences at the molecular level. In addition, microsatellites or SSR consist of variable numbers of tandemly repeated units and represent a class of repetitive DNA commonly found in eukaryotic genomes [77]. These markers are characterized by a great abundance [15, 62], a high variability [26, 70] and a large distribution throughout different genomes [40, 63, 76]. Microsatellites are typically multi-allelic loci since more than five alleles per locus are commonly observed either in plant or in animal populations [36, 41, 73]. In addition, automated PCR procedures, which enable high-throughput data collection and good analytical resolution at a low cost, have been developed for microsatellites [37, 42]. Hence, taking into account advantages of these methods, we have designed their use in

date-palms to assess genetic distances and to survey evolutionary genetic diversity.

## **MATERIALS AND METHODS**

### **Plant Material**

A set of Tunisian accessions collected from three main oases, namely: Tozeur, Gabès and Kébili were used in the study. These consisted of cultivars and male trees. Varieties were chosen for their good fruit quality and are the most common genotypes cultivated in the main plantations located in the South of Tunisia (Fig. 1). Other fruit characteristics and morphological traits for most varieties were reported by Rhouma [59, 60]. Among these varieties, the two that were recently introduced in Tozeur groves (“Zehdi” from Iraq and “Ghars Mettig” from Algeria) are also included in the study.

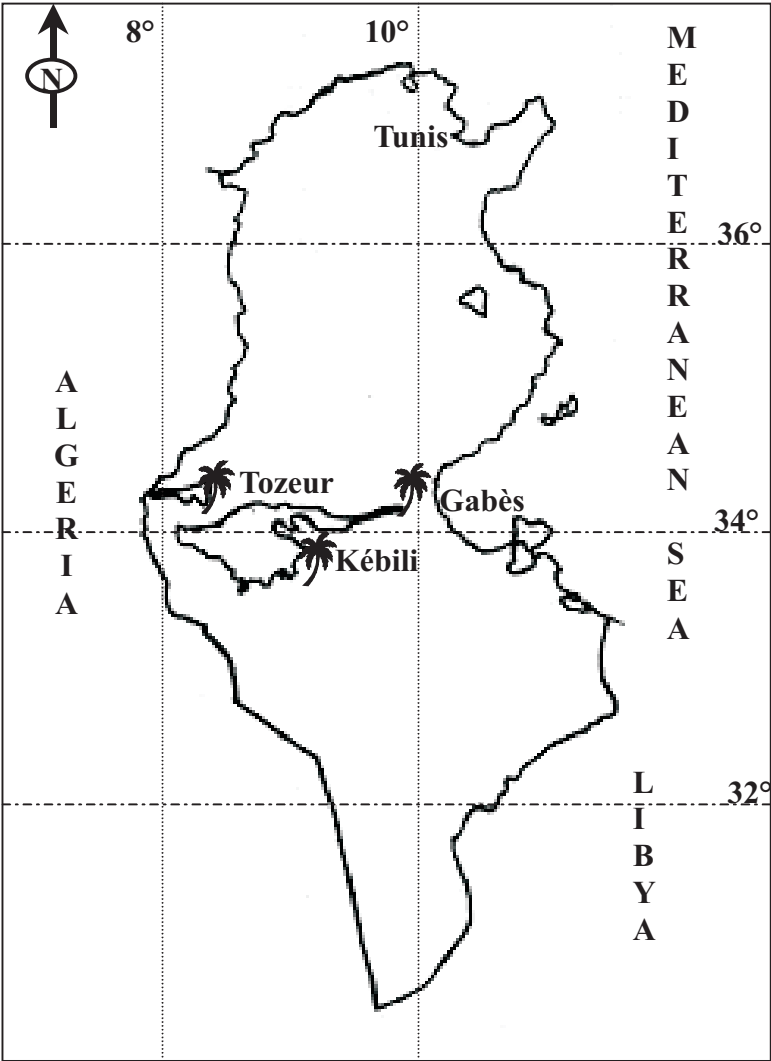
The plant material consists of young leaves from adult trees randomly sampled from the mentioned oases.

### **DNA Preparation**

Total cellular DNA was isolated and purified from frozen young leaves according to the procedure of Dellaporta et al. [18]. After purification, DNA concentrations were determined using a GeneQuant spectrometer (Amersham-Pharmacia Biotech, France) and its quality was checked on analytic agarose minigel electrophoresis according to Sambrook et al. [68].

### **Primers and RAPD Assays**

Universal random primers purchased from Operon Technologies (Alameda, USA) were tested to generate RAPD banding profiles from total cellular DNA used as templates. For PCR amplification, DNA templates (25 ng) from each sample were tested in a total volume of 25  $\mu$ l containing 50 pM of primer, 200 mM of each dNTP (DNA polymerization mix, Pharmacia), 2.5  $\mu$ l of enzyme buffer and 1.5 unit of Taq DNA polymerase (QBIogene, France). The reaction mix was overlaid with a drop of mineral oil to avoid evaporation during the cycling. PCR reactions were carried out with a Crocodile III thermal cycler (QBIogène, France) programmed to execute: 1 cycle at 94°C for



**Fig. 1.** Tunisia map showing the geographic origin of the analyzed date-palm ecotypes. Scale 1/100 000

5 minutes, followed by 25 cycles, each consisting of a denaturation step at 94°C for 30 seconds; an annealing step at 52°C for 1 minute and an elongation step at 72°C for 1 minute. A 5 minutes final extension was performed at the end of the last PCR cycle.

## Primers and ISSR Assays

A total of 12 primers, shown in Table 2, were tested to amplify DNA banding patterns using the varieties' total cellular DNA.

PCR reaction mixture (25  $\mu$ l) is composed of: 20–30 ng of total cellular DNA (1  $\mu$ l), 60 pg of primer (1  $\mu$ l), 2.5  $\mu$ l of Taq DNA polymerase reaction buffer, 1.5 unit of Taq DNA polymerase (QBIOfène, France) and 200  $\mu$ M of each dNTP (DNA polymerization mix, Amersham-Pharmacia, France). Each reaction mixture was overlaid with 25  $\mu$ l of mineral oil to avoid evaporation during PCR cycling. Amplifications were performed in a Crocodile III thermal cycler (QBIOfène, France). The apparatus was programmed to execute the following conditions: a denaturation step of 5 minutes at 94°C, followed by 35 cycles each composed of 30 seconds at 94°C, 90 seconds at the primer's specific melting temperature ( $T_m$ ) (Table 2), and 90 seconds at 72°C. A final extension step of 5 minutes was run at the end of the last PCR cycle.

To reduce the possibility of cross contamination and variation either in the RAPD or ISSR amplification batches, master mixes of the reaction constituents were always used and a negative control, reaction mix without any DNA was also included.

After amplification, the reaction mixture was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV light [68].

## SSR Genotyping

A total of 16 date-palms specific primer pairs developed by Billotte et al. [10] were tested in the present study. SSRs were screened on Li-Cor IR2 automated DNA sequencer (Li-Cor, Lincoln, NE, USA) by loading 0.2  $\mu$ l of PCR product diluted 10  $\times$  in loading buffer, on 6.5% polyacrylamide gel. Automatic genotyping and allele size scoring were performed by the SAGA-GTTM software (Li-Cor, Lincoln, NE, USA).

## Data Analysis

For each primer, the total number of bands and the polymorphic ones were calculated. Data were then transformed into a binary matrix where the presence of the generated band is scored as 1 and its absence is

notified by 0. A genetic distance matrix was estimated from the data matrix by using the Genedist (version 3.572c) programme based on the formula of Nei and Li [48]. The genetic distance matrix was then analyzed with the Neighbour program to produce a dendrogram using the Unweighted Pair Group Method with the Arithmetic Averaging Algorithm (UPGMA) cluster analysis [74], or using the Neighbour-Joining (NJ) algorithm [24]. Bootstrap values were computed over 100 replications using MSA 3.10 [19]. The TreeView program was used to draw the between and among accessions' dendrogram. Appropriate programs in PHYLIP (Phylogeny Inference Package, version 3.5c) and TreeView (Win32, version 1.5.2) were used to carry out all these analyses [25,53]. The subsequent ISSR and RAPD's similarity matrices obtained with ISSR and RAPD were compared according to the Pearson's and Spearman's correlation coefficients using the Statistical Analysis System Version 6.1.2 software [69].

In addition, the sampling was subdivided into three geographical groups of cultivars (female), and a group composed of the male individuals. For each group of date-palm genotypes, the genetic diversity was estimated by the determination of the allelic diversity (total number of alleles, and allele frequencies per group), the observed and expected heterozygosity ( $H_{\text{obs}}$  and  $H_{\text{exp}}$ ) [49]. The total genetic diversity ( $H_t$ ), the mean genetic diversity within population ( $H_s$ ) and the genetic differentiation among populations ( $G_{st}$ ) were also calculated using the program Genetix 4.04 [5]. The allelic richness, corresponding to the evaluated number of alleles independently from the sample size, was assessed using FSTAT 2.9.1 [31]. A Wilcoxon-Mann-Whitney test was applied to differentiate the allelic richness scored values using Statistica 6.0.

Data were also computed using GENEPOP 3.1 [56] and FSTAT 2.9.1 programs to test pairwise linkage equilibrium (LE) at all loci over the four groups to calculate  $F_{is}$  and pairwise estimates of  $F_{st}$  according to the formula of Weir and Cockerham [81].

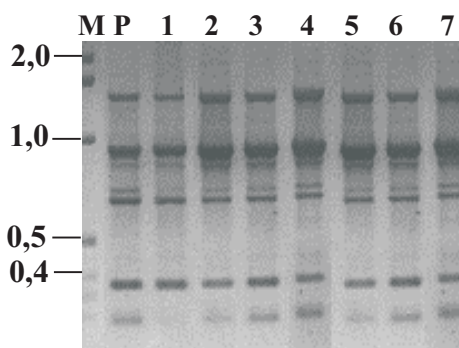
In order to compute ordinations and hierarchical classifications, the Populations 1.2.28 Software of Langella [39] was used to calculate the shared allele distance: DAS [14]. This genetic distance is known to be appropriate for recently diverged populations [29].

## Establishment of the Cultivars' Identification Key

Ecotypes identification was performed as follows: genotypes were hierarchically organized according to the greater number of alleles per locus. Accessions were then classified and those of similar fingerprints were grouped.

## RESULTS

A preliminary experiment was performed starting from total cellular DNA to generate RAPD and ISSR patterns. Due to logistical constraints, only five primers that generate banding profiles over independent PCR runs were chosen to analyze variation levels in each variety. These primers were tested in presence of DNAs used as templates and extracted from different trees of two varieties. As shown in Fig. 2, the RAPD generated fragments are uniformly observed between the different palm trees of a given variety. In this case, the OPA-10 primer was used to generate the patterns from diverse trees of Deglet Nour variety. This result suggests that no variation is scored at the intra-variety level. Hence, it is assumed that each date-palm tree sampled from any variety is characterized by a consensus profile presenting substantial monomorphic DNA bands and corresponding to the variety pattern. This finding is strongly supported since date-palms are clonally propagated throughout by offshoots naturally produced by the parent tree



**Fig. 2.** RAPD banding patterns generated from diverse trees of Deglet Nour variety using the OPH-07 primer. Lanes 1 to 7: samples, P: mother plant, M: molecular standard size (1 Kb Ladder, GIBCO-BRL, France). DNA bands are in bp.

[45, 50]. Moreover, multilocus SSR genotypes concur with this assumption since two accessions of the Deglet Nour variety originated from Kebili, and Tozeur oases have exhibited similar fingerprints [84].

### **Cultivars' Identification Key**

For each date-palm accession, the detected genotypes for mPdCIR78, mPdCIR85 and mPdCIR25 microsatellites loci are scored. A total of 25 alleles are identified in these loci: 10 alleles labelled (a1 to a10) for the mPdCIR78 locus, 8 alleles denoted (b1 to b8) for the mPdCIR85, and 7 alleles (c1 to c7) for the mPdCIR25 locus. Taking into consideration the identified alleles, we have established an ecotypes' identification key (Fig. 3). This precise diagram confirmed our assumption about the Deglet Nour accessions and those of Arichti and Rochdi since they presented identical genotypes. In fact, these cultivars sampled from different oases exhibited identical fingerprints taking into account the microsatellite loci examined. This assumption is in agreement with the clonally propagation mode in date-palms [45, 50]. Consequently, the constructed identification key helped to unambiguously discriminate 47 over 47 accessions studied (100%). This result confirms the ability of microsatellites to fingerprint genotypes. Varietal identification keys have been previously reported in date-palms based on isozymes [11, 52] and organellar DNA haplotypes [66]. These authors have reported 69, 93 and 71% resolving power values, respectively. Therefore, our results produced neutral molecular markers powerfully suitable in cultivars' identification. In fact, a maximum of 55,440 theoretically different multilocus genotypes could be distinguished taking into account only the three mentioned loci (i.e. mPdCIR78, mPdCIR85 and mPdCIR25).

### **Genetic Diversity Analysis as Inferred by RAPDs**

Starting from total cellular DNAs extracted from the accessions studied, a large number of reproducible and polymorphic bands were obtained and scored as different RAPD markers. Depending on the DNA origin primer combination, 9 to 15 reproducible bands were evidenced in the 0.2-2.5 kb size range. A maximum of 15 bands were generated using the OPA-15 and OPM-05 primers, while only 10 fragments were produced with the OPA-01 and OPM-15 primers.

A genetic distance matrix based on the evidenced RAPDs expressed the large genetic diversity within the varieties studied since the scored



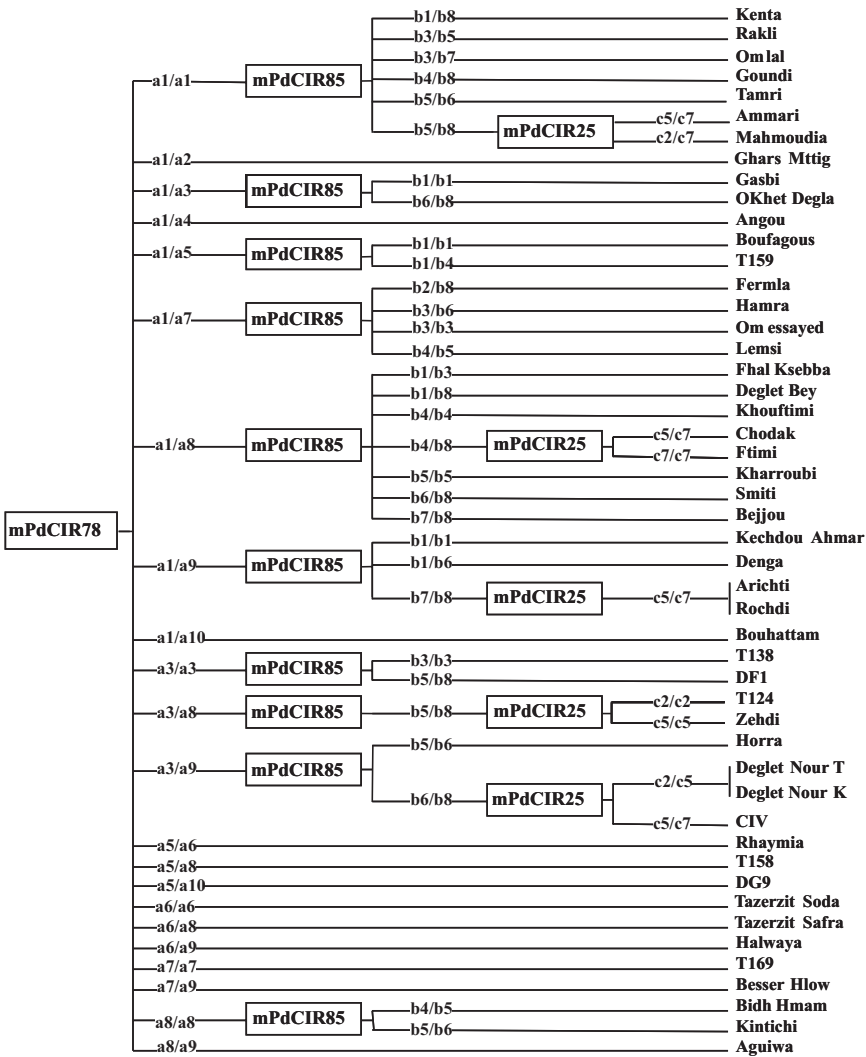


Fig. 3. Diagram illustrating the varietal identification key of 48 Tunisian date-palm ecotypes based on multilocus genotypes.

average genetic distance ranged from 0.15 to 0.86. Thus, it is assumed that the RAPD procedure is an efficient method for the detection of the date-palm polymorphism at the DNA level. The smallest distance value of 0.15 was scored either between Boufagous and Deglet Hassen, Cheknet Etterzi and Khad Khadem, or Cheknet Etterzi and Chekent

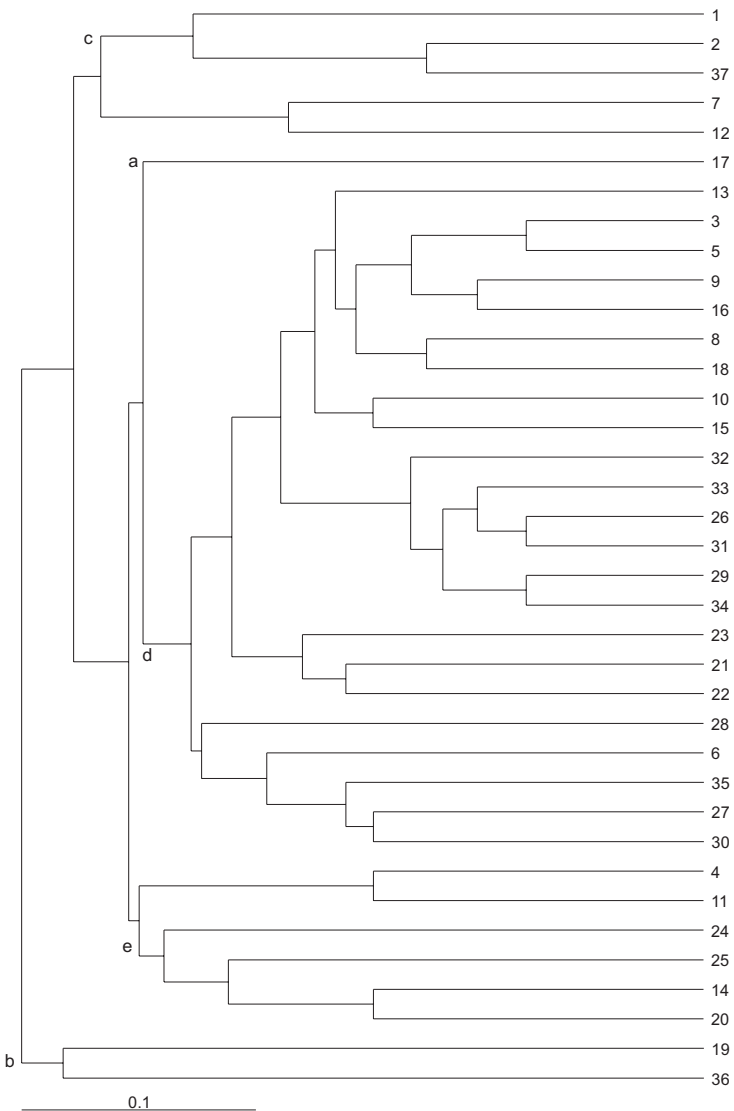
Ebbay Hamed varieties. These appear to be the most similar varieties and are closely clustered. However, Deglet Nour, Chekent Ebbay Hamed and Hlaoui varieties that have presented the greatest genetic distance of 0.86 are characterized by the maximum divergence at the DNA level. All the remaining varieties displayed intermediate levels of similarity.

## **Phylogenetic Relationships as Revealed by RAPD Data**

In order to draw the precise relationships between the genotypes tested, the genetic distance matrix was computed using the Neighbour UPGMA algorithm. The distinctiveness of the clusters identified in the derived phenogram exhibits five main cluster groups. The first one, labelled **a**, is composed of a single variety (i.e. Bouaffar). The Besser Hlou and Tazerzit Soda varieties are clustered in the second group, labelled **b**. All the remaining genotypes are clustered in the other groups, labelled **c**, **d** and **e**, that present auxiliary ramifications (Fig. 4).

## **Genetic Diversity as Inferred by ISSRs**

Among the primers screened for their ability to generate consistently banding patterns and to assess polymorphisms in the tested varieties, only seven have revealed polymorphic and unambiguously scorable bands (Table 1). These ranged from 7 to 11 with a mean of 9.57 polymorphic DNA bands per primer in a ranging size from 200-2,500 bp. A minimum of 7 and a maximum of 11 DNA fragments were obtained using (CT)<sub>10</sub>A and (AG)<sub>10</sub>C, respectively. All the remaining primers generated an intermediate number of polymorphic DNA bands suggesting that the ISSR procedure constitutes an alternative approach suitable for the date-palm DNA diversity examination. This is strongly supported by the large number of polymorphic DNA bands (i.e. a total of 67 out of 95) produced, which is higher than those observed in other cultivated crops, such as grapevine where 35 polymorphic bands were generated among closely related germplasm in presence of 12 ISSR primers [44]. In addition, the registered collective rate value of resolving power (Rp) is 43.02 with a mean of 6.14 (Table 1). Similar Rp values have been reported in lupin germplasm collection [28], potato cultivars [55], and figs [67]. Moreover, since the (AG)<sub>10</sub>G, (AG)<sub>10</sub>C and (AG)<sub>10</sub>T primers presented the highest Rp rates, it is assumed that these oligonucleotides mainly contribute in the accessions' characterization and to examine the genetic diversity in this crop.



**Fig. 4.** UPGMA dendrogram of 37 Tunisian date-palm varieties constructed from Nei & Li genetic distance based on 54 RAPD markers. The scale indicates the relative genetic distance.

1: Deglet Nour; 2: Okht Deglet; 3: Deglet Hassen; 4: Deglet Bey; 5: Boufagous; 6: Khou Boufagous; 7: Ftimi; 8: Khou Ftimi; 9: Kenta; 10: Kentichi; 11: Goundi; 12: Okht Goundi; 13: Ammari; 14: Zehdi; 15: Hlaoui; 16: Lagou; 17: Bouaffar; 18: Om Laghlex; 19: Besser Hlou; 20: Ghars Mettig; 21: Horra; 22: Angou; 23: Arichti; 24: Irhaymia; 25: Khadhraoui; 26: Darbouzi; 27: Bidh Hmam; 28: Khalt Saad; 29: Khalt Ali Meskine; 30: Tronja; 31: Khad Khadem; 32: Cheknet Hanene; 33: Cheknet Etterzi; 34: Cheknet Ebbay Hamed; 35: Tazerzit Safra; 36: Tazerzit Soda; 37: Gasbi

**Table 1.** ISSR bands and resolving power (Rp) rates of the used primers in Tunisian date-palm ecotypes

Primer sequence	Tm Optimum	Amplified bands		Rp
		Total	Polymorphic	
(AGG) <sub>6</sub>	55°C	13	10	6.61
(TGGA) <sub>5</sub>	55°C	0	-	-
(ACTG) <sub>4</sub>	45°C	6	0	-
(GACA) <sub>4</sub>	45°C	0	-	-
(GACAC) <sub>4</sub>	55°C	10	0	-
(AG) <sub>10</sub>	55°C	Smear	-	-
(AG) <sub>10</sub> G	60°C	13	10	9.46
(AG) <sub>10</sub> C	60°C	12	11	7.37
(AG) <sub>10</sub> T	57°C	12	10	7.15
(CT) <sub>10</sub> A	57°C	7	7	2.86
(CT) <sub>10</sub> G	60°C	10	9	4.84
(CT) <sub>10</sub> T	57°C	12	10	4.73
Total		95	67	43.02

The binary data matrix was computed to estimate the genetic distances between accessions. These ranged from 0.23 to 0.98 with a mean of 0.54, suggesting a high degree of genetic diversity at the DNA level (Table 2). The smallest distance value of 0.23 was observed between DF1 and T169, indicating that these two ecotypes are the most similar. The maximum distance value (0.98) is scored between Besser Hlou/Deglet Nour, Angou/Kenta, Bouhattam/Deglet Bey, Lemsi/Zehdi, Lemsi/Khou Ftimi, Lemsi/Horra, Lemsi/T138 Lemsi/T158, Denga/Horra, Gasbi/T138, and Gasbi/T158. This result suggests that the above mentioned varieties are characterized by great divergence.

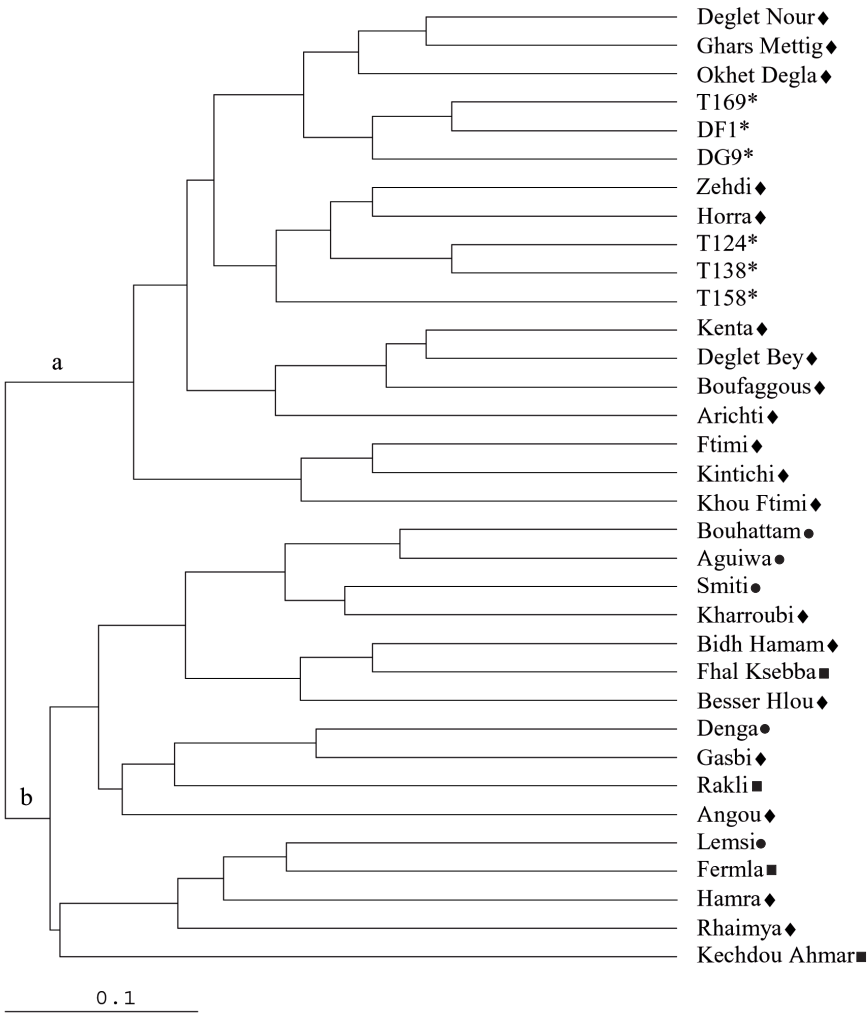
**Phylogenetic Relationships as Revealed by ISSR Data**

The genetic distance matrix was computed with the Neighbour program using the UPGMA algorithm in order to cluster the accessions according to their genetic similarity and to draw the relationships between the tested accessions. The derived dendrogram supported a varietal clustering made independently of the trees' sex and the ecotypes geographic origin and exhibited two major clusters, labelled **a** and **b**

Table 2. Genetic distance matrix among 34 Tunisian date-palm ecotypes based on ISSR data and estimated from Nei & Li's formula

Ecotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1 Deglet Nour																																	
2 Boufagous	0.47																																
3 Ftimi	0.37	0.43																															
4 Kenta	0.47	0.28	0.43																														
5 Kintichi	0.61	0.61	0.31	0.61																													
6 Deglet bey	0.37	0.31	0.40	0.26	0.50																												
7 Ghars mettig	0.26	0.37	0.40	0.37	0.50	0.29																											
8 Zehdi	0.34	0.40	0.43	0.47	0.61	0.43	0.31																										
9 Arichri	0.54	0.47	0.50	0.40	0.78	0.37	0.37	0.34																									
10 Khoutimi	0.54	0.69	0.37	0.53	0.40	0.57	0.50	0.50	0.53																								
11 Horra	0.43	0.50	0.47	0.73	0.37	0.50	0.53	0.31	0.65	0.37																							
12 Okhet degla	0.31	0.43	0.53	0.43	0.57	0.40	0.34	0.37	0.57	0.5	0.34																						
13 TI24	0.47	0.34	0.57	0.53	0.61	0.50	0.37	0.34	0.53	0.61	0.37	0.31																					
14 TI38	0.61	0.69	0.73	0.53	0.69	0.57	0.50	0.47	0.61	0.53	0.43	0.43	0.47																				
15 TI158	0.53	0.53	0.73	0.53	0.69	0.50	0.43	0.34	0.47	0.61	0.37	0.43	0.23	0.28																			
16 TI69	0.43	0.65	0.78	0.50	0.82	0.40	0.47	0.65	0.65	0.57	0.61	0.40	0.50	0.50	0.43																		
17 DF1	0.26	0.65	0.61	0.57	0.65	0.47	0.34	0.50	0.57	0.43	0.53	0.47	0.57	0.50	0.50	0.23																	
18 DG9	0.28	0.61	0.65	0.47	0.61	0.50	0.37	0.47	0.69	0.40	0.50	0.43	0.47	0.61	0.53	0.31	0.31																
19 Lemsi	0.69	0.87	0.82	0.78	0.78	0.92	0.82	0.98	0.87	0.98	0.98	0.73	0.87	0.98	0.98	0.73	0.65	0.78															
20 Bouhattam	0.73	0.92	0.69	0.73	0.73	0.98	0.69	0.82	0.73	0.82	0.92	0.87	0.73	0.73	0.73	0.61	0.78	0.65	0.43														
21 Smiri	0.69	0.78	0.50	0.78	0.47	0.65	0.57	0.61	0.53	0.69	0.65	0.65	0.78	0.78	0.69	0.65	0.73	0.87	0.73	0.43													
22 Dengra	0.57	0.73	0.69	0.73	0.82	0.61	0.61	0.73	0.65	0.92	0.98	0.78	0.92	0.82	0.73	0.78	0.78	0.82	0.43	0.34	0.57												
23 Hamra	0.43	0.65	0.69	0.65	0.65	0.69	0.53	0.65	0.65	0.73	0.69	0.69	0.57	0.73	0.65	0.61	0.47	0.50	0.43	0.61	0.50	0.61											
24 Aguiwa	0.65	0.65	0.61	0.65	0.73	0.98	0.69	0.82	0.73	0.65	0.69	0.69	0.82	0.73	0.73	0.53	0.69	0.65	0.57	0.28	0.31	0.47	0.61										
25 Kharroubi	0.78	0.87	0.73	0.69	0.53	0.73	0.65	0.87	0.61	0.69	0.73	0.65	0.61	0.69	0.61	0.65	0.82	0.87	0.87	0.43	0.34	0.65	0.73	0.43									
26 Angou	0.69	0.69	0.82	0.98	0.61	0.65	0.57	0.69	0.78	0.61	0.57	0.73	0.61	0.61	0.47	0.57	0.50	0.69	0.87	0.73	0.69	0.57	0.92	0.65	0.61								
27 Besser Hlou	0.98	0.78	0.65	0.78	0.69	0.92	0.73	0.78	0.53	0.61	0.73	0.73	0.69	0.69	0.69	0.57	0.65	0.78	0.61	0.50	0.34	0.73	0.73	0.31	0.47	0.78							
28 Rhainia	0.65	0.65	0.69	0.50	0.57	0.69	0.69	0.73	0.73	0.73	0.87	0.78	0.92	0.92	0.82	0.47	0.47	0.73	0.50	0.61	0.57	0.53	0.61	0.47	0.57	0.65	0.50						
29 Bidh-bham	0.57	0.50	0.78	0.57	0.57	0.61	0.53	0.65	0.57	0.65	0.69	0.53	0.57	0.73	0.65	0.47	0.40	0.50	0.73	0.61	0.57	0.61	0.53	0.47	0.50	0.43	0.43	0.40					
30 Gasbi	0.54	0.61	0.73	0.69	0.87	0.65	0.65	0.69	0.61	0.87	0.82	0.82	0.87	0.98	0.98	0.82	0.65	0.69	0.61	0.65	0.78	0.37	0.82	0.50	0.53	0.53	0.69	0.50	0.57				
31 KchdouAhmar	0.61	0.61	0.73	0.53	0.53	0.78	0.73	0.73	0.61	0.78	0.78	0.82	0.73	0.78	0.69	0.61	0.57	0.50	0.78	0.61	0.50	0.61	0.92	0.82	0.65	0.61	0.69	0.65	0.50	0.78			
32 Fhalkebbba	0.69	0.61	0.82	0.69	0.78	0.73	0.50	0.61	0.40	0.53	0.65	0.50	0.40	0.61	0.53	0.65	0.65	0.53	0.87	0.73	0.61	0.82	0.65	0.50	0.47	0.34	0.65	0.31	0.47	0.78			
33 Rakli	0.54	0.61	0.73	0.40	0.61	0.65	0.50	0.61	0.61	0.69	0.73	0.50	0.61	0.87	0.61	0.73	0.82	0.47	0.61	0.50	0.69	0.50	0.73	0.43	0.47	0.61	0.78	0.73	0.57	0.50	0.69	0.53	
34 Fernla	0.47	0.69	0.57	0.53	0.53	0.82	0.57	0.87	0.87	0.87	0.69	0.82	0.73	0.61	0.78	0.69	0.57	0.61	0.40	0.37	0.50	0.65	0.50	0.69	0.87	0.61	0.43	0.73	0.87	0.47	0.87	0.69	

(Fig. 5). This interpretation is well illustrated in the case of the cluster **a** that includes either varieties or male ecotypes. Furthermore, the cluster **b** is composed of varieties originating from the different date-palm oases.



**Fig. 5.** UPGMA dendrogram of 34 Tunisian date-palm accessions constructed from Nei & Li genetic distance based on 67 ISSR markers. The scale indicates the relative genetic distance. Accessions originated from Tozeur, Gabès and Kébili oases are labelled (◆), (●) and (■) respectively. Males are labelled with an asterisk.

## Genetic Diversity and Phylogenic Relationships as Inferred by Combined RAPD and ISSR Data

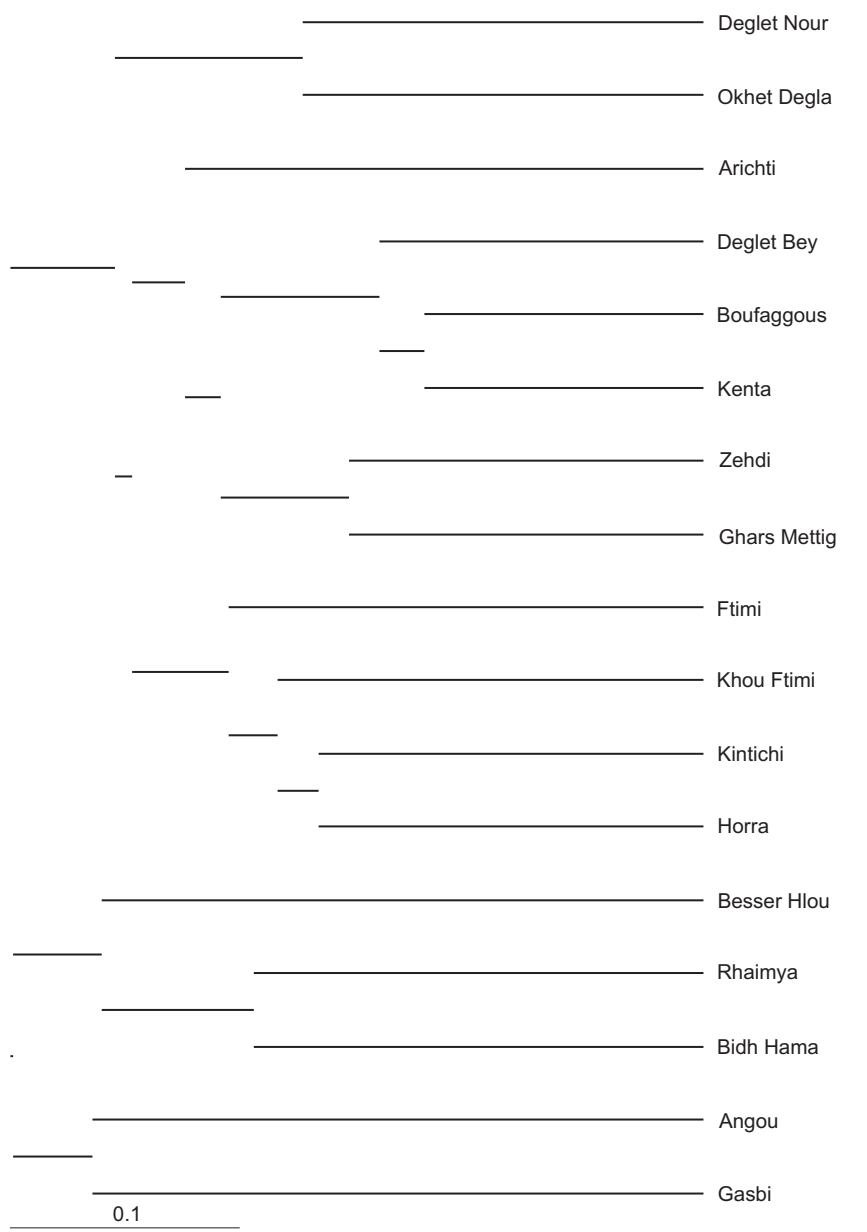
Since slight differences are denoted in the RAPD and the ISSR dendrograms, RAPD data were taken into account together with the ISSRs in order to identify the relationships between a subset of 17 varieties. A total 102 polymorphic bands (54 RAPDs and 48 ISSRs) were used to examine the genetic polymorphisms in this crop. The derived genetic distance matrix based on the Nei and Li's formula and reported in Table 3 exhibited values ranging from 0.29 to 0.82 with a mean of 0.53. The smallest genetic distance of 0.29 is scored between Boufagous and Deglet Bey varieties suggesting their great similarities at the DNA level. However, the Deglet Nour and Besser Hlou, the Okhet Degla and Besser Hlou seem to be the most dissimilar varieties, as the genetic distance registered between them is the highest.

The derived dendrogram illustrated in Fig. 6 based on RAPD markers together with ISSRs, permitted to cluster the varieties studied in two main groupings. The first one is composed of Besser Hlou, Rhaimya, Bidh Hmam, Angou and Gasbi varieties. All the remaining ones are clustered in the second group that exhibited three subgroups. As reported above, this typology clusters the varieties based on their nomenclature and/or fruit parameters. This dendrogram's topology is also almost identical to this, based on the ISSR data either by the number of clusters, or by their composition (data not shown). However, a few differences have been scored between dendrograms based on RAPD data and the combined RAPD/ISSR. This result suggests that the designed methods contribute differently in the discrimination of the date-palm varieties. This assumption is strongly supported since polymorphisms obtained with RAPD and ISSR markers have different underlying sources at the molecular level and may differ in their informativeness for the exploration of genetic diversity and the establishment of relationships between ecotypes [22]. Hence, the determination of the contribution of each technique in the observed diversity is of a great interest in order to determine whatever they contribute in the Tunisian date-palm polymorphisms. This could be made possible by estimation of the correlation indices between the three obtained genetic distances matrix. As reported in Table 4, the Pearson and Spearman coefficients exhibited high positive and significant correlations between RAPD data matrix and the combined data (Pearson's coefficient = 0.2629,  $r_P = 0.00$ ; Sperman's

**Table 3.** Genetic distance matrix among 17 Tunisian date-palm cultivars based on RAPD and ISSR data and estimated from Nei & Li's formula

Cultivar	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 DegletNour																
2 OkhetDegla	0.35															
3 Deglet Bey	0.48	0.48														
4 Boufeggous	0.48	0.42	<b>0.29</b>													
5 Ftimi	0.47	0.47	0.51	0.45												
6 Khou Ftimi	0.60	0.44	0.48	0.42	0.38											
7 Kenta	0.48	0.48	0.27	0.24	0.48	0.51										
8 Kintichi	0.65	0.51	0.53	0.41	0.36	0.36	0.53									
9 Zehdi	0.51	0.42	0.50	0.44	0.51	0.55	0.47	0.47								
10 BesserHlou	<b>0.82</b>	<b>0.82</b>	0.64	0.71	0.73	0.62	0.64	0.71	0.67							
11 Ghars Mettig	0.44	0.56	0.33	0.42	0.50	0.53	0.36	0.51	0.31	0.62						
12 Horra	0.64	0.50	0.48	0.39	0.50	0.38	0.58	0.33	0.39	0.65	0.47					
13 Angou	0.71	0.80	0.55	0.55	0.60	0.50	0.73	0.55	0.73	0.69	0.50.	0.44				
14 Arichti	0.62	0.58	0.44	0.47	0.55	0.51	0.47	0.64	0.44	0.67	0.45	0.51	0.51			
15 Rhaimya	0.71	0.64	0.58	0.58	0.67	0.56	0.55	0.58	0.62	0.55	0.60	0.60	0.64	0.69		
16 Bidh Hmam	0.51	0.55	0.44	0.41	0.62	0.55	0.47	0.50	0.56	0.50	0.39	0.51	0.42	0.50	0.39	
17 Gasbi	0.53	0.50	0.62	0.51	0.60	0.64	0.58	0.69	0.65	0.73	0.64	0.67	0.53	0.62	0.50	0.62





**Fig. 6.** UPGMA dendrogram of 17 Tunisian date-palm cultivars constructed from Nei and Li's genetic distance estimated on 102 combined RAPD and ISSR data. The scale indicates the genetic distance.

coefficient = 0.1684,  $rP = 0.00$ ). Negative, but not significant, correlations were obtained between ISSR and RAPD on the one hand and ISSR/RAPD on the other hand, supporting the groupings' differences observed in dendrograms based on RAPDs and the RAPD/ISSR combined data. Therefore, it may be assumed that ISSRs are more informative in terms of discriminating date-palm varieties rather than the RAPDs. Differences in the distribution of these classes of markers throughout the genome constitute a critical factor in determining their efficiency in the investigation of the genetic variability of this crop. This is in agreement with data reported in other plant species [22, 38, 47]. Moreover, other studies have described a faster evolution rate of the neutral ISSR markers and an association of RAPD bands with functionally important loci [22, 54].

**Table 4.** Pearson's coefficient (upper diagonal) and Sperman's coefficient (lower diagonal) based on ISSR RAPD and ISSR/RAPD data \*: significative value (0.05)

Marker	ISSR	RAPD	ISSR/RAPD
ISSR	1.0000	-0.1754	-0.1754
RAPD	-0.1468	1.0000	0.2629*
ISSR/RAPD	-0.1468	0.1684*	1.0000

### Genetic Diversity as Inferred by SSR Data

Out of the 16 primer pairs tested for their ability to generate expected SSR banding patterns in Tunisian date-palms, 14 have successfully established the ecotypes' genotypes. The SSR profiles exhibited more than four different alleles per locus with clearly identifiable homozygous and heterozygous genotypes. A total of 100 alleles with a mean of 7.14 alleles per locus were scored (Table 5). The number of alleles per locus varied from 4 (mPdCIR16) to 10 (mPdCIR78).

$H_{exp}$  values (Table 6) ranged from 0.40 (mPdCIR35) to 0.83 (mPdCIR85) indicating that the Tunisian date-palm collection is characterized by a high degree of genetic diversity. The alleles' number, as well as their frequencies, varied significantly among the Tunisian subgroups. This is well exemplified in the case of the mPdCIR90 locus exhibiting seven alleles in Tozeur oasis, and four out of them have not been evidenced in the remaining subgroups. Moreover, the mean number

**Table 5.** Summary of 14 microsatellite loci revealed in the Tunisian date-palm genotypes studied

<i>Locus</i>	<i>Alleles</i>		<i>Genotypes</i>
	<i>Size</i>	<i>Number</i>	
mPdCIR10	142 - 181	8	16
mPdCIR15	142 - 157	7	11
mPdCIR16	148 - 156	4	7
mPdCIR25	219 - 250	7	14
mPdCIR32	302 - 318	8	16
mPdCIR35	200 - 214	5	7
mPdCIR50	172 - 222	8	19
mPdCIR57	260 - 288	8	15
mPdCIR63	139 - 171	5	8
mPdCIR70	205 - 227	7	15
mPdCIR78	138 - 173	10	22
mPdCIR85	175 - 197	8	21
mPdCIR90	162 - 193	7	12
mPdCIR93	181 - 202	8	18
Total		100	201

of alleles varied from one group to another (Table 7). However, there is no significant difference in allelic richness among the four groups ( $P=0.15$ ). This result agrees with multilocus means of expected heterozygosity ( $H_{exp}$ ) and unbiased heterozygosity ( $H_{nb}$ ) that did not significantly differ among groups at  $P>0.05$  (Table 7).

As reported in Table 8, it is assumed that for all the loci studied, the  $H_s$  and  $H_t$  values are nearly similar, suggesting that the maximum variability is locally maintained. This assumption is confirmed by the low values of  $G_{st}$ . For instance, the multilocus values of  $H_s$ ,  $H_t$  and  $G_{st}$  are 0.6449, 0.6885 and 0.0633, respectively. Moreover, these results suggested that only 7% of the genetic diversity is explained at the inter-group level, while 93% of this variability is maintained at the intra-group level.

In addition, estimation of  $F_{is}$  values, according to the formula of Weir and Cockerham [81], indicates that the Tozeur group as well as the male genotypes showed a significant deviation from Hardy-Weinberg Equilibrium (HWE). Results of the exact test [64] and the specific test

**Table 6.** Expected ( $H_{exp}$ ) and observed ( $H_{obs}$ ) Heterozygosity in each group by locus computed using GENETIX

Locus		Tozeur	Kébili	Gabès	Males	All accessions
mPdCIR10	$H_{exp}$	0.75	0.42	0.81	0.54	0.73
	$H_{obs}$	0.67	0.60	1.00	0.57	0.69
mPdCIR15	$H_{exp}$	0.71	0.64	0.40	0.62	0.68
	$H_{obs}$	0.70	0.40	0.50	0.43	0.59
mPdCIR16	$H_{exp}$	0.60	0.66	0.61	0.49	0.60
	$H_{obs}$	0.67	0.60	0.83	0.57	0.67
mPdCIR25	$H_{exp}$	0.73	0.58	0.57	0.70	0.72
	$H_{obs}$	0.83	0.60	0.83	0.14	0.71
mPdCIR32	$H_{exp}$	0.76	0.82	0.78	0.66	0.77
	$H_{obs}$	0.67	1.00	0.67	0.86	0.71
mPdCIR35	$H_{exp}$	0.45	0.32	0.28	0.24	<b>0.40</b>
	$H_{obs}$	0.33	0.00	0.33	0.00	0.26
mPdCIR50	$H_{exp}$	0.75	0.78	0.74	0.65	0.75
	$H_{obs}$	0.77	0.60	0.83	0.71	0.73
mPdCIR57	$H_{exp}$	0.71	0.66	0.74	0.50	0.71
	$H_{obs}$	0.67	0.20	0.67	0.29	0.57
mPdCIR63	$H_{exp}$	0.64	0.42	0.28	0.72	0.63
	$H_{obs}$	0.23	0.60	0.33	0.14	0.26
mPdCIR70	$H_{exp}$	0.71	0.78	0.72	0.72	0.75
	$H_{obs}$	0.30	0.20	0.00	0.29	0.26
mPdCIR78	$H_{exp}$	0.77	0.68	0.72	0.78	0.79
	$H_{obs}$	0.73	0.80	1.00	0.57	0.73
mPdCIR85	$H_{exp}$	0.83	0.80	0.78	0.76	<b>0.83</b>
	$H_{obs}$	0.73	0.80	1.00	0.57	0.75
mPdCIR90	$H_{exp}$	0.69	0.58	0.61	0.49	0.68
	$H_{obs}$	0.67	0.80	0.83	0.57	0.69
mPdCIR93	$H_{exp}$	0.80	0.70	0.72	0.72	0.80
	$H_{obs}$	0.90	0.60	0.83	0.86	0.86

for heterozygote deficiency (U test [64]) strongly supported this finding since statistically significant deficits for the mentioned groups have been registered. However, no deviation from HWE is observed in the two remaining groups (i.e. Gabès and Kébili) (Table 7).

**Table 7.** Genetic diversity indices for the four groups

Group	$H_{exp}$	$H_{nb}$	$H_{obs}$	$F_{is}$	$P$ value	Mean Number of alleles/locus
Tozeur	0.71	0.72	0.63	0.1222	0.0000	6.79
Kébili	0.63	0.70	0.56	0.2258	0.0983	4.00
Gabès	0.63	0.68	0.69	-0.0140	0.7789	4.00
Males	0.62	0.66	0.47	0.3083	0.0001	3.71
All accessions	0.70	0.71	0.61	0.142	0.0000	7.14

**Table 8.** The total genetic diversity ( $H_t$ ), the mean genetic diversity within population ( $H_s$ ) and the genetic differentiation among groups ( $G_{st}$ ) estimated using the program Genetix 4.04

Locus	$H_s$	$H_t$	$G_{st}$
mPdCIR10	0.6294	0.6851	0.0813
mPdCIR15	0.5945	0.6307	0.0574
mPdCIR16	0.5901	0.6039	0.0229
mPdCIR25	0.6456	0.7123	0.0937
mPdCIR32	0.7540	0.7940	0.0504
mPdCIR35	0.3239	0.3346	0.0320
mPdCIR50	0.7299	0.7556	0.0340
mPdCIR57	0.6525	0.7093	0.0801
mPdCIR63	0.5163	0.5734	0.0996
mPdCIR70	0.7333	0.7697	0.0473
mPdCIR78	0.7367	0.8034	0.0831
mPdCIR85	0.7914	0.8374	0.0549
mPdCIR90	0.5933	0.6594	0.1003
mPdCIR93	0.7378	0.7703	0.0422
Multilocus	0.6449	0.6885	0.0633

**Phylogenetic Relationships as Revealed by Microsatellite Markers**

A derived NJ dendrogram based on Das genetic distance, exhibited three main clusters, each one composed of males as well as cultivars (Fig. 7). The two Deglet Nour and the two Arichti accessions, which originated from different date-palm oases, are strongly clustered and suggest that

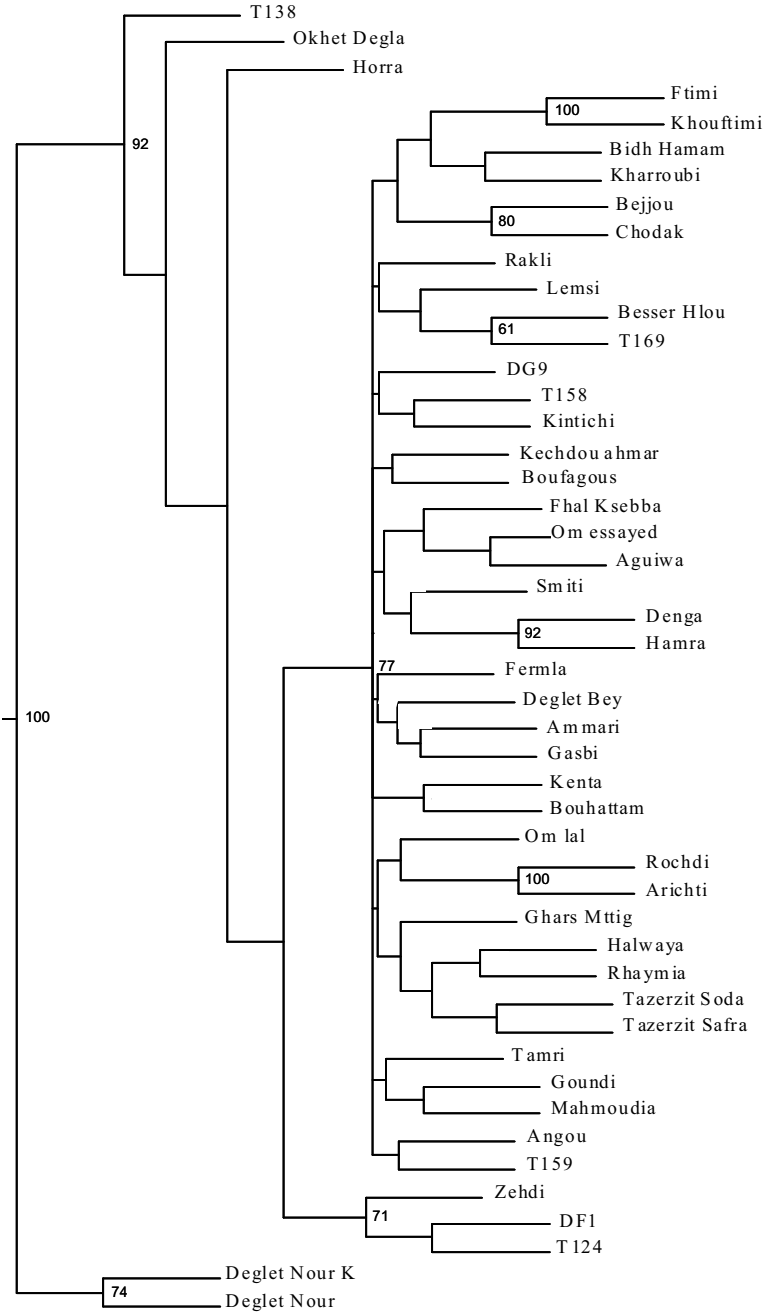
**Table 9.** Pairwise comparisons of the multilocus Fst values

Group	PopT	PopM	PopG	PopK
Tozeur	0.0000			
Males	0.0095	0.0000		
Gabès	0.0202	0.0492	0.0000	
Kébili	-0.0219	-0.0144	-0.0193	0.0000

these cultivars correspond to similar genotypes. In addition, the observed clustering topology showed that groupings of accessions are made independently either from their geographic origin or the sex of trees. This result is corroborated by the absence of geographic structure in the plotting of accessions on the two first PCO axes (data not shown). Moreover, pairwise comparisons of the multilocus Fst values scored among the pre-established groups were not significant ( $P>0.05$ ), indicating that all groups revealed high genetic affinity (Table 9).

**CONCLUSION**

The objective of this study was to characterize a large number of Tunisian date-palm ecotypes with the help of the RAPD, ISSR and SSR markers in order to investigate their phylogenetic relationships. The designed procedures have enabled the survey of the DNA polymorphism in the collection analyzed. On the whole, our results concur with those describing the use of RAPD technique in date-palms starting from Moroccan, Tunisian, Saudi and Iraqi collections [3, 6, 72, 79]. These authors assumed that the studied accessions are clustered independently of their geographic origin and suggested a narrow genetic diversity in this crop. Both analyses have generated a dendrogram topology, which is in agreement with those based on morphometric criteria particularly related to the fruit parameters [59]. This is well exemplified in the case of Boufaggous and Deglet Bey varieties characterized by nearly similar dates (large size and dark colour). In addition, accessions' groupings are not well defined either according to their geographical origin or the sex of trees, since the introduced varieties and the male ecotypes did not significantly diverge from the autochthonous female accessions. Therefore, data derived from the evidenced markers (RAPDs, ISSRs and SSRs) suggest that all the date-palm ecotypes are interrelated in spite of their phenotypic divergence. A common and narrow genetic basis is



**Fig. 7.** Dendrogram of Tunisian date-palm ecotypes based on *Das* genetic distance estimated from microsatellite data.

strongly supported by selection applied by farmers based either on the fruit quality or the ecotypes' adaptation to local conditions. Hence, a small part of the genome that encodes interesting agronomic parameters is affected by this selection in the natural populations from which clones originated. Consequently, it may be assumed that the data strongly supported the ancient date-palm's Mesopotamian "Fertile Crescent" domestication origin [30, 45, 83, 86].

Compared to the diversity reported in other fruit crops, the overall polymorphism exhibited in the present study is rather high, suggesting that the designed methods are very effective in assessing the molecular polymorphism of this crop. In addition, the revealed SSR alleles were successfully used to discriminate the studied ecotypes since an identification key has been established on the basis of three microsatellite loci. In this case, the data provided higher percentage of resolution than that scored in date-palms using either isoenzymes or plastid haplotypes [8, 9, 11, 52, 66]. The transfer of SSR analysis to other laboratories over the world would be of great interest to label at a large scale offshoots, any other plant material at early stage and *in vitro* plantlets. A precise fingerprinting of unlimited number of these closely related ecotypes reported across the world could be made possible on the basis of our procedure. Such a strategy could improve cultivars' differentiation and then contribute to their labelling (homonymy and synonymy) since large numbers of ecotypes have been reported in the date-palm' growing countries [1, 7, 43, 61, 71]. Moreover, since offshoots' exchanges are currently occurring, the evidenced SSR markers are greatly recommended to be used as descriptors in the certification and the control of original labels of date-palm material originated from these countries.

A deeper insight of the reported markers could constitute a powerful tool to molecularly characterize the genetic diversity of this crop and trace parentage and genetic relationships within closely related genotypes at the specific level and below. This can be made possible through analysis of natural hybrids between date-palm and its relatives from the *Phoenix* species reported in Algeria, Morocco, Punjab and Senegal [45]. Research is presently being carried out to specify the phylogenic and the culture origins of the date-palms [46] and to shed light on the domestication process in this phyto-genetic resource.



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## Oil Palm

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### ABSTRACT

Palm breeders have made significant increases in the genetic yield potential of oil palm since breeding began systematically at the beginning of the 19<sup>th</sup> century, with an estimated four-fold increase in yields during this period [22]. This represents a doubling of yields due to genetic improvement and a further doubling due to improved agronomic practices.

One of the major breeding developments was the recognition of the genetic control of shell-thickness by a single gene [3]. This alone increased yield by 25% with a switch from planting thick shelled (dura) fruited trees to the thinner shelled hybrid (tenera). This switch has also had a fundamental effect on the way in which oil palm is bred and improved, as all commercial material must now be of the tenera shell-type and is essentially a hybrid between the dura mother palm and the pisifera pollen palm.

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It will be a major challenge to continue to generate significant improvements in the genetic material used for palm oil production in the coming years (both for economic and environmental benefits).

In the first section of this article, the background to the current success is examined, both in terms of the biology and breeding of oil palm. The second part of the article deals with how biotechnology has and will effect the genetic improvement of oil palm.

**Key Words:** *Oil palm, breeding, molecular markers, crop improvement*

## INTRODUCTION

### Botanical Classification and Phylogeny

#### *Palms*

Palms are woody monocotyledons belonging to the family *Arecaceae* (an alternative name to *Palmae*), in the order *Arecales* [61]. They are a natural group of plants with fossil records dating from the late Cretaceous, and with a characteristic appearance. The present evidence also suggests that palms probably evolved very early in the history of the monocotyledons. Phylogenetic analysis of monocot relationships based on plastid *atpB* (chloroplast gene encoding the  $\beta$  chain of ATP synthetase), *rbcL* (the large subunit of RUBISCO), *matK* (gene located within the intron of chloroplast gene *tmK*), and *ndhF* (chloroplast gene), mitochondrial *atpA* (gene encoding the  $\alpha$  chain of ATP synthetase) and nuclear 18S and 26S rDNA (ribosomal DNA) [12] placed *Arecales* as a sister-group to the rest of Commelinids, which includes orders *Commeliniales*, *Zingiberales* and *Poales*. However, *Arecales* (single family *Arecaceae*, the palms) were not included in the Commelinids although Dahlgren *et al.* [24] noted that the palms shared some characteristics with those families and the connection of *Arecaceae* to *Pandanaceae* and *Cyclantaceae* based on similarities in habit and inflorescence indicate parallelism rather than close phylogenetic relationship.

There are no morphological synapomorphies (shared, derived state) for the palms overall. However, phylogenetic studies of *rbcL* [11, 28]), 18S rRNA [46, 108], and the chloroplast gene *rps4* [83], all support monophyly of the palm family [114]. The latest phylogenetic analysis of *Arecaceae* [47] added sequence data of 51 genera of Arecoid Line for plastid genes (*atpB*, *rbcL* and *ndhF*) and the plastid intergenic spacers (*tmT* and *tmQ-rps16*). Furthermore, Hahn [48] added the nuclear DNA

(18S rDNA) and chloroplast DNA (cpDNA; *atpB* and *rbcL*) sequence for 65 palm genera. The detailed extensive array of molecular phylogenetic studies of palms has identified four major groups corresponding to: (1) Calamoideae, (2) Nypoideae, (3) Coryphoideae + Caryoteae, and (4) Arecoideae. The Arecoid line is the largest of the four groups with approximately 60% of the genera in the family. The analysis of biogeographic patterns present in Arecoid line suggests that the group is of Gondwana origin [47].

Palms are evolving slowly at the sequence level. Studies of *rbcL* and comparative studies of chloroplast DNA RFLPs revealed that palm plastid genomes have an approximately five-fold slower rate of synonymous substitution compared to other monocots, especially grasses [39, 118]. This difference in rates of divergence has been confirmed with the nuclear *Adh* gene ([40, 82]. Studies of variation in palm mitochondrial genes have been focused on the gene *atpA* [32]. Results showed that the nuclear genes evolve faster than the chloroplast genes, and the chloroplast genes evolve faster than the mitochondrial genes as shown previously by Wolfe *et al.* [119]. The relative rates of divergence within groups seem consistent, but palms evolve at synonymous sites more slowly than grasses.

The patterns of non-synonymous substitutions are different; only *rbcL* and *Adh1* evolve significantly faster in grasses compared to palms. The rates of nucleotide substitution in the nuclear ribosomal small subunit (18S nrDNA) are significantly lower than that seen in other monocots, and comparable to those of the plastid gene *rbcL* [108].

In the palm family there is little variation in chromosome number, but the genome size can vary significantly. Differences in chromosome numbers are unusual and polyploidy is also rare in palms [113].

### **The Elaeidinae**

The oil palm (*Elaeis guineensis* Jacq.) belongs to the subfamily Arecoideae, tribe Cocoeae and subtribe Elaeidinae. The analysis of biogeographic patterns present in tribe Cocoeae suggests that the tribe is of Gondwana origin and primary diversification in this group may have coincided with continental breakup [47]. The subtribe Elaeidinae includes only the genus *Elaeis* (from the Greek *elaia*, for the olive tree) and *Barcella*, and is always recovered as monophyletic [47]. The genus *Barcella* has no commercial use at present. The genus *Elaeis* consists of



only two species: (1) the African oil palm - *Elaeis guineensis* Jacq., and (2) the Latin American oil palm - *Elaeis oleifera* Cortez [22].

Mitochondrial DNA has been used to assess the phylogeny of the subtribe *Elaeidinae* to which the African and Latin American oil palm and the genus *Barcella* belong [2]. The authors analyzed 288 representative accessions of *Elaeis oleifera* and 38 of *Elaeis guineensis*, by performing RFLP with four mitochondrial probes. The RFLP analysis identified more mitotypes in *E. oleifera* compared to *E. guineensis*, and also confirmed that the divergence between the two species was very low.

### ***Elaeis guineensis* – the African oil palm**

There are no subspecies in *Elaeis guineensis* Jacq. However, there is a range of breeding populations of restricted origin (BPROs) such as Pobe, Yangambi, Deli Dura, AVROS and others, documented by Rosenquist [100], which play an important role in many breeding programmes. One of the most important BPROs is the Deli Dura, which is believed to have descended from four palms that were planted in 1848 in the Bogor Botanical Gardens, Indonesia. Concern has already been raised about the limited within population genetic diversity for some BPROs such as Deli Dura. (ref. section 1.6).

*Elaeis* species have 16 pairs of chromosomes [71]. On the basis of their length, the chromosomes of *Elaeis guineensis* were divided into three groups [71]. The size of the haploid *E. guineensis* genome has been estimated to be  $1.7 \times 10^9$  bp (basepairs) ( $2C=3.7$  pg (picograms)) [97].

### **Geographical Distribution**

Although the two *Elaeis* species occur on separate continents and have different growth habits, they are very similar. However, while they can hybridize to produce some fertile offspring, the differences between them are sufficient to treat them as separate species. Barcellos *et al.* [2] have proposed the centre of origin of the genus *Elaeis* to be Latin America, based on their results which revealed more variability in *E. oleifera* than in *E. guineensis* accessions examined by them, and on botanical evidence.

However, the fossil, historical and linguistic evidence (particularly from Brazil) for the African origin of *E. guineensis* is strong. Zeven [122] reported finding fossil pollen similar to *E. guineensis* from Miocene and

earlier layers in the Niger delta and there have been similar results obtained [30, 31] [96]. Zeven suggested that *Elaeis* sp. originated in Africa and spread to South America via the Tertiary bridges, which are believed to have connected Africa and America. Both results from Africa and South America are consistent with a initial *Elaeis* species, which underwent geographical speciation with the breakup of Gondwana Land, some 60 million years ago, without the need for a specific mechanism to prevent cross-fertilization of the derived species (although fertility is a major issue with the interspecific F1) [53].

The first historical evidence of oil palm cultivation in Africa comes from the Portuguese explorer Ca' Da Mosto (1434-1460; [23] although the species was recorded in detail by Jacquin [58].

It is generally accepted that the present geographical distribution of oil palm is the reflection of favourable climate and of human farming activities with palms almost certainly being spread by the migration of man [22].

## **Biology of Oil Palm**

### ***Inflorescence, flower and fruit structure***

Separate male and female inflorescences arise in the leaf axils among the leaves, and the infructescence is large and densely packed with fruit primordia. Approximately two inflorescences are initiated per month and take up to three years to develop into a mature male or female inflorescence. The oil palm is monoecious, alternately producing male and female inflorescences in a cycle of around six months, and is thus naturally out-crossing. Detailed studies of the flowers have, however, shown that each flower primordium is a potential producer of both female and male organs, though one or the other almost always remains rudimentary [50]. The female inflorescence is a panicle consisting of a variable number of rachillae that carry 5 to 30 floral triads, each composed of one female flower accompanied by 2 non-functional male flowers. Although each female flower has a tricarpellate ovary, only one carpel usually develops to give rise to a seed. The ovary is accompanied by two rudimentary androecia. In male inflorescences each male rachilla is composed of 400-1,500 staminate flowers. In very rare cases (tissue culture stress, or very young plants) both androecium and gynoecium

develop to give rise to a hermaphrodite flower. Inflorescence abnormalities are by no means uncommon in oil palm. The sex of the inflorescence is influenced by the external conditions for about two years before anthesis and also by a genetic component [20]. The cycle between male and female inflorescences can be biased towards male inflorescences under harsh external conditions, such as drought, and towards female inflorescences under favourable external conditions.

It was originally believed that oil palm, because of its abundant pollen and a reduced flower structure, was wind pollinated. In fact, the introduction of the pollinating insect *Elaiedobius kamerunicus* into Malaysia from Africa showed that insect pollination plays an essential part in the fruit set, especially in wet conditions [51].

The seed (kernel) and the pulp (mesocarp) of the fruit are very rich in oil. In the internal fruit structure the most important differences are to be found in the thickness of the shell (endocarp). There three known fruit forms are: (1) *dura*, (2) *pisifera* and, (3) *tenera*, which were determined by Beinaret and Vanderveryen [3] to be due to a single gene. The importance of this gene is well understood, because only plants of intermediate type (*tenera*) are grown commercially. In the *pisifera* form (*shsh*) there is no shell (endocarp) as such, only a fibrous ring. In the *dura* form (*ShSh*) there is a thick endocarp, and in the intermediate *tenera* form (*Shsh*) the endocarp is thinner and the fibrous ring is present too. The thickness of the endocarp varies considerably in the *dura* and *tenera* forms, the distributions even overlap, so the ultimate criterion for distinguishing *dura* from *tenera* is the presence of the fibrous ring in the *tenera*. There are no fruit types as such recognised in *Elaeis oleifera*, all fruit appear to be of *dura* form.

The external appearance of a fruit varies when ripened. There are four major fruit types known: (1) *nigrescens*, (2) *virescens*, (3) *albescens* and (4) *poissoni*. The fruit is normally dark and is called *nigrescens*. A relatively uncommon type is *virescens*. Its green colour is due to the absence of anthocyanin in the exocarp of the fruit and the colour changes at maturity to orange. The white colour of the mesocarp (*albescens*) is caused by the absence of, or low level of, carotenoids in the mesocarp. *Poissoni* is an abnormal fruit type which is often referred to as 'mantled', or as 'a fruit with supplementary carpels'.

## Oil Palm Products

Oil is the main commercial product of the oil palm. The oil is extracted from the fruit mesocarp (palm oil) and nut kernels (kernel oil). Palm oil, extracted from the fibrous flesh of fruits (mesocarp) after they have been hot-squeezed, has oil content from 40–70%. Prime oil, commercially known as palm kernel oil, is extracted from the seeds.

About 90% of world's palm oil is used for edible purposes [102]. Numerous studies have shown the association between diet and the incidence of coronary disease (CHD). Palmitic acid (44%) is the major saturated fatty acid in palm oil and this is balanced by almost 39% monounsaturated oleic acid and 11% polyunsaturated linoleic acid. The remaining acids are largely stearic (5%) and myristic (1%). This composition is significantly different from palm kernel oil (obtained as a co-product during the processing of oil palm fruits), which is almost 85% saturated, short chain ( $C_{12} - C_{14}$ ), lipids. Nutritional studies showed that diets with a high proportion of palm oil are as healthy as any other diet, because the fat component is equivalent to that of other edible oils [22].

The fatty acid composition of palm oil ( $\approx 1:1$  saturated to unsaturated fatty acids) is such that the oil is semi-solid at normal room temperature ( $22^{\circ}\text{C}$ ), which favours its use as the solid-fat component for margarine. More liquid oils need to be treated by hydrogenation to make them solid, leading to trans-fatty acids [115]. Palm oil is particularly suitable for deep-frying because of low content of polyunsaturated linoleic acid and a higher level of saturated fatty acids [102], which are less susceptible to oxidation. In addition, palm oil contains natural antioxidants such as tocopherols and carotenoids. During the last few years interest has been evinced in red palm oil as a source of vitamin A in human nutrition [109, 121].

### ***Palm oil based oleochemicals, diesel and biodegradable plastics***

Only about 10% of palm oil is used for non-food products such as oleochemicals e.g. sodium salts (soaps) and glycerol [70]. Furthermore, fatty acids and methyl esters from palm oil can be used as substitutes for diesel [15]. Alcohol can be also produced by fermentation of carbohydrates [22]. Biodegradable plastics such as polyhydroxybutyrate (PHB) could be synthesized from acetyl coenzyme A, the precursor for fatty acid-synthesis by transforming oil palm [55, 74].

## **Other products from oil palm**

One of the major ‘waste’ products from the oil palm mill is the empty fruit bunches which are left after the fruit is steam-stripped from the fresh fruit bunches. These are ideally used as fuel, and occasionally as mulch on young field planted palms [22]. After oil extraction, the remaining palm kernel material can be pressed to form fodder cake for cattle. Kernel cake from screw press machines has 8-13% residual oil which can make a useful contribution in animal diets [22]. The wood from palm trunks is relatively soft and is only really useful for forming pressed wooden objects [65, 67].

## **Oil Palm Crop Improvement**

### ***Advances in the crop improvement of oil palm***

A more scientific approach to crop improvement began towards the middle of the 20th century, when Beinaret and Vanderveryen proved that the *tenera* form was generated by crossing *dura* and *pisifera* forms. This has been the single most important step in the genetic improvement of oil palm yields. The thick-shelled *dura* form is homozygous for one allele (ShSh), but has a yield disadvantage of about 25% compared to the thin-shelled heterozygous *tenera* form (Shsh) (commercially grown type), while the shell-less *pisifera* is homozygous for the alternative allele (shsh), but is often female-sterile, and cannot be grown as a crop.

The oil palm is monoecious and is naturally cross-pollinated. Although both parents have male and female flowers, for seed production the *dura* form is used as a female parent and *pisifera* has taken the role of the male parent.

Progress to date in oil palm breeding to increase oil yields has been spectacular, with a four-fold increase in yield over the last century [19]. Half of this has been ascribed to improvement in the genetic material. Within Deli Dura, a comparison of unselected material (grown under the best agronomic practices) derived from Bogor Botanical Gardens, Indonesia, and material after four generations of selection, suggested a 50% increase in yield, largely from an increase in mesocarp/fruit [19]. Hardon *et al.* [49] has estimated that improvement in yield per generation has been 10-15%, although this only equates to approximately 1% per year. In general, two approaches have been adopted for oil palm

breeding. The basic approaches are Family and Individual Selection (FIS), and Reciprocal Recurrent Selection (RRS). FIS identifies the best families and then selects the next generation of parents from within these, using mainly phenotypic values. Such an approach resembles animal breeding [33]. RRS aims to develop separate and complementary pools of Pisifera and Dura which exploit hybrid vigour when crossed. These are progeny tested against each other before further development, allowing the generation of breeding values. This is the method favoured by maize breeders.

Rosenquist [100] suggested that some of the disadvantages of RRS were the tendency to produce inbreeding within the maternal and the paternal pools of material, as well as the more limited numbers of palms in the base population. In practice, many programmes are a combination of the two approaches, as oil palm has the advantage over maize of being perennial, with palms having good breeding values making repeated contributions to breeding material, and the female sterility of many pisifera sources (such as AVROS) makes progeny testing necessary.

However, recent results from Dami dura, suggest that inbreeding within the maternal pool is no longer an over-riding concern [26]. This might raise a doubt whether many of the deleterious recessive genes have already been eliminated through the RRS programme.

It is difficult to see how such impressive increases in oil yield can be maintained in future generations, without a major contribution from biotechnology.

## **Molecular Markers in Oil Palm Breeding**

The primary objective of oil palm research is to increase profit per hectare from plantations; [17]. Breeding and selection of *Elaeis guineensis* began in the early 1920s and since then considerable improvements have been made both in yield and quality characters [51, 100]. The potential yield of the crop may be as high as 17 tons of oil per hectare [17]. The long breeding cycle and the variation still encountered suggest that there exists considerable scope for improvement in yield [17], disease resistance [36] and oil composition. Molecular markers represent one way in which it may be possible to select within material earlier in the breeding cycle to reduce the generational times.

## ***Fingerprinting and linkage studies***

Linkage mapping is frequently performed using polymorphic DNA markers such as isozymes [41, 42] and RFLPs (Restriction Fragment Length Polymorphism; [77]). RFLPs may be generated by gain or loss of restriction sites and/or indels between restriction sites. Jack *et al.* [56] and Mayes *et al.* [77] reported the potential in oil palm for marker identification and application and the use of highly informative oil palm RFLP markers for genotype characterization Mayes *et al.* [77, 78] and Jack *et al.* [57] reported construction of a RFLP map for oil palm and subsequent identification of a marker linked to shell thickness. There were 24 linkage groups identified (resolved to 21 by Rance *et al.*, [95]) although oil palm has only 16 chromosomes. 40 RFLP markers have also been used for estimating genetic similarity within oil palm breeding parents such as Deli, AVROS etc. [79].

RFLPs are gradually being replaced by less laborious and more polymorphic marker systems which are based on PCR, such as SSRs (Simple Sequence Repeat; [4, 110]) and AFLPs (Amplified Fragment Length Polymorphism; [116]). These systems are generally used for saturating already existing RFLP maps. Kulartne *et al.* [68] used AFLP markers for studying the diversity within different populations collected by Malaysian Palm Oil Board (MPOB). Purba *et al.* [92] investigated the genetic relationships between genotypes from different *E. guineensis* populations used in IOPRI (Indonesian Oil Palm Research Institute) breeding programme with the help of AFLP markers. The findings indicated that the crosses among African sub-populations were more potential for breeding than the crosses between the African and the Deli populations currently used in the reciprocal recurrent selection. An AFLP map with 20 linkage groups was reported by Chua *et al.* [16]. Microsatellites (or SSRs) are small arrays of tandem repeats that are simple in sequence (e.g.  $[CA]_{10}$ ). SSRs are thought to have been produced by mutation, unequal crossing-over and DNA slippage. Microsatellites are neutrally evolving, co-dominant markers with high levels of genetic diversity and show Mendelian inheritance.

About 400 microsatellite markers (SSRs) were recently developed in the *E. guineensis* species by CIRAD (Centre de coopération internationale en recherche agronomique pour le développement), employing a microsatellite-enriched library building procedure from a

hybridization-based capture methodology using biotin-labelled microsatellite oligoprobes and streptavidin-coated magnetic beads [4]. The SSR polymorphism was characterized in the *E. guineensis* and in the closely related species *E. oleifera*, in which utility of the SSR markers was observed, as well as on a subset of 16 other palm species to which some oil palm SSRs were potentially transferable [5]. Transferability of some date palm and peach palm SSRs in the *Elaeis guineensis* species was also observed [6, 7]. A reference linkage map of oil palm was developed in the control cross LM2T × DA10D, using 944 loci (255 SSR, 688 AFLP, locus *Sh*) distributed on 16 linkage groups representing the 16 chromosome pairs of the oil palm ([8], <http://tropgenedb.cirad.fr/oilpalm/publications.html>). Two AFLP markers were located on this map at 7 cM and 11 cM on each side of the *Sh* locus controlling the variety type of the fruit in oil palm, using bulk segregant analysis and linkage mapping methods. A further 103 SSRs were also developed by Mayes and co-workers ([90] PIPOC (International Palm Oil Congress); <http://www.gen.cam.ac.uk>).

A range of other marker systems is available, such as RAPDs (Random Amplified Polymorphic DNA; [117] and ISSR (Inter Simple Sequence Repeats, [123]. RAPDs use random sequence 10 nucleotide primers, and amplification of products depends on the presence of the complementary nucleotide sequence in the opposite orientation on each DNA strand within a stretch of less than approximately 3 Kbp (kilobase pairs). They are rapid, simple but often not reproducible and not transferable between laboratories. In ISSR, primers anchored at the 3' end that anneal to microsatellites are used. Shah [104] assessed the utility of RAPD markers for determination of genetic variation in oil palm and Moretzsohn [81] produced a RAPD linkage map of the shell thickness locus in oil palm. Rajanaidu *et al.* [93] used RAPDs and RFLPs to estimate genetic diversity and compare different populations collected by the MPOB.

There have been a number of Long-Terminal-Repeat Retrotransposon (LTR-RTN)-based marker systems utilizing retrotransposons, such as SSAP (Sequence Specific Amplified Polymorphism), IRAP (Inter Retrotransposon Amplified Polymorphism) and REMAP (Retrotransposon Microsatellite Amplified Polymorphism) developed in plants [91]. In the case of LTR RTN-based markers, the unique biological process of retrotransposition generates polymorphisms,



which is an irreversible process resulting in insertions of RTNs into new sites without the loss of the parental copies. The consequences of retrotransposition range from the alteration of a few hundred base pairs to a few kilobases at the site of insertion. By contrast, marker systems employing simple sequence repeats (SSRs) are based on random small-scale changes (i.e. from one up to a few tens of nucleotides). Price *et al.* ([91], <http://www.gen.cam.ac.uk>) developed a multilocus IRAP marker system based on *copia* - like retrotransposons. The authors also reviewed marker methods based on retrotransposons and concluded that there was scope for using these methods in oil palm breeding and diversity analysis as an alternative to AFLP.

### **Potential applications of markers—simple traits**

Development of markers would shorten the process of selection, especially at the nursery stage. Currently, crossing and growth in the nursery stage take approximately two years. After two years, seedlings are field planted (typically 143 palms per hectare). Although the seedlings start to produce fruit after 2.5 years, 5 years of recorded field data is necessary to properly assess the quality of a potential breeding palm. Marker-assisted selection (MAS) would accelerate the speed of the process. Some of the important areas of interest are shell-thickness, *virescens* and crown disease. A marker for shell thickness could have a potentially high commercial value in breeding programmes. The value would be in determining whether selected palms are *pisifera* or sterile *tenera* before progeny testing. This marker could also assess purity of commercial *tenera* seed lots [77]. Hartley [50] states that *virescens* is possibly monofactorial and dominant. Exploitation of this gene, if a segregating population were available, could make spotting ripe bunches much easier and thus contribute to a higher yield, through decreasing the loss of loose fruit.

The development and establishment of technologies, such as MAS [80], which would allow selection of individuals to be based on the genetic marker information, would represent a major step in the oil palm breeding.

Markers would also assist breeding programmes by: (1) helping to maintain diversity within the populations used for breeding, (2) identifying outcrosses in breeding programmes, and (3) allowing

controlled introduction of foreign material into breeding programmes. MAS would be used in conjunction with the existing selection based on General Combining Ability (GCA), Specific Combining Ability (SCA), and other breeding values.

It has been noted by Corley and Tinker [22] that the progress made in the breeding programme depends both on the amount of variation present in the population before the selection starts, and on the heritability of the characteristics to be selected by the breeder. It is worth pointing out that the majority of characters measured by oil palm breeders are likely to be polygenic and these include bunch yield and its components; oil and kernel to bunch and their components; and carotene content. Although the major effect on bunch composition is the shell thickness gene, the attempts made to find a marker for this gene were not entirely successful (the linkage was not sufficiently close) [5, 78, 81], until the recent development of the Billotte map (Link2palm EU Framework 6 programme). Recently there has been an interest in high carotene content [93], for its increased nutritional value. It has also been shown that in the crosses derived from the Nigerian material the carotene content ranged from 180 to 2,500 ppm (parts per million).

The systematic approach of extensive phenotypic markers and GCA analysis would allow the association of markers and phenotypic characters and enable quantitative trait loci (QTL) analysis.

### ***Potential applications of markers—complex traits***

Marker based methods could provide a means of using QTL (Quantitative Trait Loci) analysis to target regions of a genetic map and measuring their effects. Linkage mapping of QTL depends on detecting the linkage disequilibrium between marker regions involved and the trait genes themselves. Localization of a QTL depends more on the population size than on the density of markers [66], as well as on the heritability of the trait studied. Firstly, the problem with the QTL mapping in oil palm is that population sizes are small and the basis of the genetic heritability of many quantitative traits is yet to be determined. The number of individuals in any one controlled cross is often limited (<90). Secondly, QTL are more easily identified for inbred lines but they are much more difficult to identify in out-crossing species where there is much more background variation. Thirdly, for parents to provide linkage

information, the hybrid F1 must be heterozygous at both a marker and a linked QTL because only in this case can marker-trait associations be made in the progeny.

The availability of a large number of published SSR markers and dominant marker systems such as AFLP, REMAP and IRAP, and the relative ease with which those systems can be converted to automated marker typing, should allow rapid identification of markers linked to agronomically important traits and subsequent QTL analysis.

The first attempts at QTL analysis in oil palm were reported by Rance *et al.* [95]. The authors investigated the underlying genetic basis of quantitative traits (QTL) in oil palm and identified six marker regions associated with QTL effects; RFLP markers were identified linked to yield, oil/bunch and its components, and vegetative characters.

The recent development of the CIRAD genetic map enables a detailed QTL analysis as the populations reach maturity. Already, an initial analysis has identified a QTL for palm height (palm2LINK).

### **Potential applications of markers—disease resistance**

The most serious diseases are *Fusarium* wilt (*Fusarium oxysporum* f.sp.*elaeidis*) in several parts of Africa and Latin America; *Ganoderma* in Asia, dry basal rot (*Ceratocystis*) in West Africa and fatal yellowing and sudden wither on new plantations in Latin America [43]. Unsuccessful attempts at finding RFLP markers linked to *Fusarium* wilt resistance have been made by Buchanan [10]. The present availability of highly polymorphic markers such as microsatellites makes this task much more feasible. *Ganoderma* trunk rot or basal stem rot has been a problem in some areas of Malaysia and Indonesia for the last 40 years, and in recent years it has also been the subject of much research in those countries. Most of this work has been summarized by Flood *et al.* [37], which also includes a general review of the current state of this disease in Asia by Ariffin [1]. de Franqueville *et al.* [25] showed that there were significant differences between families in *Ganoderma* incidence and thus demonstrated the feasibility of breeding for resistance and of potential marker application. There has been extensive research on fatal yellowing (*Thielaviopsis paradoxa*), much of it is reviewed by Gomez *et al.* [43]. To combat this disease, markers could be used to search for disease

resistance factors within *Elaeis guineensis* material and in interspecific hybrids with *Elaeis oleifera*.

## Genome Organization

### **BAC (Bacterial Artificial Chromosome) libraries**

Plant genomes are remarkably large and dynamic and contain up to 80% of repetitive DNA [103].

BAC libraries can be constructed: (1) to represent the majority of the genome possible (restriction endonucleases such as *Hind*III and *Eco*RI, which cut frequently and show no significant sensitivity to methylation of the genomic DNA), or (2) to try to target the coding regions (rare cutting methylation sensitive restriction endonucleases, such as *Mlu*I and *Not*I). Large insert clone libraries, such as BACs [106] are essential tools. A partial BAC *Hind* III library was reported by Singh *et al.* [107] with the average size inserts of 40 kb and a complete *Hind*III oil palm BAC library has been made recently by CIRAD [87]. Complete libraries have the advantage that they contain all of the clonable sequences in the genome, but with the disadvantage that this requires very large numbers of clones and significant infrastructure and resources for development, maintenance and usage of those clones. An alternative approach is to develop 'targeted' BAC clones. These use methylation patterns within the genomic DNA to produce restriction enzyme cuts where there is no methylation present; lack of methylation is often indicative of coding and coding-associated regions [73]. The construction of an initial test BAC library for oil palm using the methylation-sensitive rare cutting restriction endonuclease, *Mlu*I has allowed this possibility to be examined (Hafeez and Mayes, unpublished). While the average insert size is relatively low (80 Kbp), hybridization of specific sequence probes to colonies [89] and analysis of 600 *Mlu*I BAC end-sequences, compared to *Hind*III derived BAC clones and end-sequence, confirm significant enrichment for low-copy sequences and the exclusion of high copy-number classes of retrotransposon from *Mlu*I clones.

Understanding something about the structure of the oil palm genome could be a major advantage in the development of targeted markers for MAS.

## Uses of Conserved Synteny

Perhaps the nearest relative of oil palm from within those major crops, which have been studied in depth, could be cereals. It is estimated that the cereal group and oil palm diverged some of 100 million years ago [12]. Closer relatives of oil palm on which marker work has been conducted, are date palm (*Phoenix dactylus*) and coconut palm (*Cocos nucifera*), and Billotte *et al.* [6] have demonstrated that SSR markers developed in one species could have been used in another. Indeed, one of the targets of the recent EU Framework 6 INCO-DEV Link2palm proposal was to cross map markers in coconut and oil palm. While this work is underway and will provide useful information, oil palm is probably the most developed of these three species in terms of molecular tools and will only make limited gains from the other two species.

An initial study testing RFLPs which were used in cross mapping between a segment of Chromosome 9 in rice with chromosome 5 in wheat [38] gave some evidence for conservation of gene order between cereals and oil palm (3 out of 5 markers mapped in rice/wheat also showed linkage in oil palm [Hafeez, unpublished], however distances between markers were considerably greater in oil palm and currently with the lack of sequence data, this approach is unlikely to make a major impact in the next few years.

## Vegetative Propagation of Oil Palm

The reasons for developing methods for vegetative production of oil palm are many, however, the drawback is that it has only one vegetative meristem and cannot be propagated by taking cuttings. The ability to rapidly propagate elite genotypes has immense potential for a species with a selection cycle of 10–16 years. Yield advantages over seedling populations were predicted in the order of 30%. In spite of the fact that attempts at propagating oil palm by tissue culture started in the 1960s, the discovery of abnormal flowering and severe bunch failure caused a major setback, just as commercial exploitation had begun [18]. Presently the application of clonal plant production in oil palm is still limited due to the occurrence of these somaclonal variants. It has been shown that the frequency of abnormal flowering varies between the clones, with some clearly being more susceptible [27]. Furthermore, Eeuwens *et al.* [29] showed that the tissue culture medium largely affected the

embryoids during multiplication: short transfer intervals between mediums and low level of auxin result in a high level of cytokinin, which increases the risk of somaclonal variation. Better protocols are being developed alongside molecular work to try to understand the basis of the change in floral morphology and methods of exploitation have also been adapted. The use of tissue culture to produce ‘clonal seed’ is seen as an intermediate step towards full commercial exploitation. Clonal seed has the advantage that one of the parents is an elite clone, while the other is a seed-derived palm (as dura palms are limiting for the production of commercial seed, the dura is often the clonal palm). This has the advantage that any recessive somaclonal mutations should be masked by the contribution of the seedling gamete.

It has been widely assumed that abnormal flowering is an ‘epigenetic’ phenomenon. A definition of epigenetics, as postulated by Russo *et al.* [101], says “epigenetics is a study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by the changes in DNA sequence”. Cytosine methylation is one of the more prevalent and intriguing mechanisms for generating epigenetic change. Whereas symmetric CpG nucleotides are the major target for methylation in animals, methylcytosine in plants and fungi can occur at C residues at symmetric (CpG; CpNpG) and asymmetric CpXpX (where X is any base other than G) sites [35, 45]. Methylation is required for the normal development of animals and plants [64] and functions as a global repressor of gene expression [9]. There is increasing evidence that reduced DNA methylation can result in abnormal plant development [14, 34]. Interestingly, the “mantled” somaclonal variation in oil palm has been shown to be correlated with DNA hypomethylation, thus indicating that normal and abnormal plants differ in the degree of methylation of nuclear DNA [59, 75]. Furthermore, Kaeppeler *et al.* [63], in the review of somaclonal variation in plants, suggested that variation in methylation levels as a result of tissue culture could possibly be a cause for the abnormal flowering.

It has been suggested that methylation evolved as a genomic defence against invasive DNA, including TEs (Transposable Elements) and viruses [120]. Although most plant TEs are not transcriptionally active (i.e. they are neutral), they can be reactivated under the conditions of abiotic or biotic stress, so-called ‘genomic stress’ [69]. However, only *Tnt1* and *Tto1* have been observed to be actually transposing [44, 54,

88]. Further application of a marker system based on retrotransposons to the oil palm somaclonal variants of the same ploidy exhibiting differences in the genome size would perhaps help in understanding the potential role of retrotransposons in the generation of somaclonal floral variants. It is worth noting that so far molecular markers have been unsuccessful in drawing out differences between normal and mantled palms, which would be sufficiently repeatable and efficient enough to be useful as a screening method (proteins – [72]; cytokinins – [62]; DNA markers – [13, 84, 105]; messenger RNA [60, 94, 98, 99, 111, 112].

## **Transformation Technology**

The first evidence of transient expression of a reporter gene (glucurodinase; GUS) in oil palm tissues delivered by microprojectile bombardment was reported in 1993 [76] and since then significant effort has gone into developing potential transformation systems for oil palm, with major focus being oil composition [86], abscission of fruit [52] and higher resistance to disease [10]. The potential of this approach to oil palm improvement was examined and reported by Corley and Stratford [21] and initial transformation of oil palm with a marker gene has been reported [85]. Corley and Stratford [21] estimate that production of a transformant line in sufficient numbers for field planting could be 15 years, even after transformation has been achieved. Given the very mixed results using transformation in annual crops (and the sensitivity in the EU (European Union) over transgenic products) it could be a couple of decades before we see major implementation of this technology for oil palm.

## **Conclusion**

### ***The future of genetic improvement in oil palm***

The impressive progress made over the last 90 years will be difficult to match in the future without a substantial contribution from marker-assisted selection and transformation and tissue culture approaches.

These offer the potential to short-circuit the long breeding and selection cycles currently needed for the genetic improvement and multiplication of oil palm, as well as offering novel solutions to genetic and agronomic problems through transformation.

## **Molecular genetics**

Recent advances in creation of generally accessible, co-dominant SSR markers by the EU Link2Palm programme (<http://www.neiker.net/link2palm/OilP/DefOIL.htm>) will provide resources for generating genetic maps that can be integrated and used for dissecting the genetic basis of some of the key traits in oil palm. Generating molecular markers for direct MAS will be one consequence of this, but possibly, equally important will be gaining knowledge of the genetic basis and inter-relations for a number of agronomic traits. This may enable modification of breeding approaches for improving their efficiency without the intrinsic use of markers and their associated costs.

Also, the creation and characterization of the first oil palm EST clone database should enable the development of the first slide-based microarrays and facilitate the first use of the potentially extremely powerful transcriptomics approach in oil palm (<http://www.mpob.gov.my/>).

Limited sequence currently exists for oil palm, but this is likely to expand rapidly and a complete genome-sequencing programme for this important oil crop can only be a matter of time.

## **Models and comparative genetics**

Using model systems to investigate oil palm has great potential, despite the considerable genetic distance between oil palm and any of the information-rich model systems. Whether the direct use of conserved gene order between species such as rice and oil palm will be feasible is unclear, although the ability to characterize genes in model systems such as *Arabidopsis thaliana* and to use information from conserved biochemical pathways will be invaluable.

Important areas where comparative genetics may make an impact include control of cell abscission in fruit, reduction in the problem of loose fruit collection, engineering of oil quality and composition through transgenic expression of homologous or heterologous oil biosynthesis genes, or even approaches to reduce height increment without a reduction in yield, as has occurred for many cereal crops with semi-dwarfing genes.



## Transgenics and tissue culture

The intrinsic potential of clonal propagation should be realized in the coming years, once concerns over abnormal flowering subside, and this should facilitate the generation of transgenic oil palm expressing specific traits to improve a number of characters and develop novel traits such as materials for bioplastics, polyhydroxybutyrates (PHBs) and polyhydroxyalkanoates (PHAs) (<http://minihelix.mit.edu/malaysia/research/me1.htm>).

Perhaps one of the most important applications of transgenic technology will be the approach to reduce pests and diseases. For a disease like *Ganoderma*, where there is variation in genetic susceptibility but the inheritance appears complex, transgenic approaches may be critical and would certainly justify the research investment and, not the least, the 15-20 year timescale needed to produce and test a transgenic oil palm.

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# **PLANT GENOME: BIODIVERSITY AND EVOLUTION**

Volume 1, Part D

**Phanerogams (Gymnosperm) and  
(Angiosperm-Monocotyledons)**

## Genome Complexity of *Allium*

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### ABSTRACT

The *Allium* genome is one of the largest among monocotyledonous plants. Understanding relationships between the Alliaceae taxa facilitates in determining the strategies to select superior germplasm used in crop improvement programs. Species hybridization broadens genetic variation. Molecular-marker technology allows portions of genomes to be targeted and exploited when designing new crops with desirable traits found in wild or related species. In order to place *Allium* species in proper taxa, the *Allium* genus has been investigated at molecular and DNA levels so that genetic differences that separate and identify the species are revealed. The objective of this monograph is to present a survey of recent biochemical, molecular, and cytogenetic approaches that have been used on alliaceae taxa so as to elucidate genome organization in *Allium*.

**Key Words:** Alliaceae, molecular markers, genome organization, chemotaxonomy, DNA technology

**Abbreviations:** AFLP = amplified fragment length polymorphism, CAPs = cleaved amplified polymorphic sequences, CMS = cytoplasmic male sterility, ESTs = expressed sequence tags, FISH = fluorescence in situ hybridization, GISH = genomic in situ hybridization, Indels = short

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insertion = deletion event, ITSs = internal transcribed spacers of nuclear ribosomal DNA, RAPDs = random amplified polymorphic DNA markers, RFLPs = restriction fragment length polymorphism, SCAR = sequence-characterized amplified region, SNPs = single nucleotide polymorphisms, SSRs = simple sequence repeats

## INTRODUCTION

Taxonomically *Allium* is a complicated genus, placed ambiguously into the families Liliaceae [80] and Amaryllidaceae by the International Code of Botanical Nomenclature. *Allium* is the most important genus of Alliaceae, nevertheless classification is problematic and not explicit [36]. Watson and Dallwitz [125] place Alliaceae into Subclass Monocotyledonae, Superorder Liliiflorae, and Order Asparagales. Others [31] follow the hierarchy of Takhtajan [113] and place Alliaceae into Class Liliopsida, Subclass Liliidae, Superorder Liliianae, and Order Amaryllidales with four subgenera: (1) *Melanocrommyum*, (2) *Rhizirideum* s. lat., (3) *Amerallium*, and (4) *Allium*. While members of the Alliaceae are morphologically varied, they share common characteristics. They are mucilaginous herbs, produce essential oils, flavonols (kaempferol and quercetin) but alkaloids are absent; they are bulbaceous or rhizomatous; mesophytic or xerophytic; leaves are aromatic (onion-scented, with allylic sulphides), simple, not evergreen, and may be alternate, distichous (leek), spiral, flat, rolled, angular, sessile or petiolate, or sheathing [125]. Early classification by morphology ([20] referenced by [78]) divided *Allium* species into seven groups. A later subdivision ([124], cited by [31]) placed cultivated *Allium* species into four sections: (1) *Cepa* (bulb onion), (2) *Phyllodolon* (Japanese bunching onion), (3) *Porum* (garlic and leek), and (4) *Rhizirideum* (chive). Subsequent classification based on morphology, crossability, and karyotype by Traub [114] divided them among the four sections: (1) *Allium*, (2) *Cepa*, (3) *Fistulosa*, and (4) *Rhizirideum*.

Of the eight independent global centers of origin established by Nikolai Vavilov, *Allium* species are found in four [123]. From Center of Origin I, Chinese, came *A. chinense* Don. (*A. odorum* L.), *A. fistulosum* L., *A. macrostemon* Bge. *A. pekinense* Prokh.; from Center III, Inner-Asian (northwestern India, Afghanistan, and Uzbekistan) came *A. cepa* L.

(sensu lato), *A. pskemense* Fedtsch., *A. vavilovii* Vved., *A. sativum* L., and *A. longicuspis* E. Regel.; from Center IV, Asia Minor, are *A. cepa* (secondary origin), *A. porrum* L., and *A. ampeloprasum* L.; and from Center VI, Abyssinian (Egypt and Somalia), *A. ascalonicum* L. arrived from Southwest Asia (Pakistan and Iran). Estimations of the number of species within the *Allium* genus vary, from 600 [125] to some 750 species [109] belonging to three subgenera and 18 sections [114]. *Alliums* are cultivated as ornamentals [51] and have been domesticated as food crops. Although most *Alliums* are edible and consumed by indigenous populations, some of the most important species are cultivated as food crops, which include *A. ampeloprasum* syn. *A. porrum* (leek), *A. cepa* (bulb onion), *A. fistulosum* (Japanese bunching or Welsh onion), *A. sativum* (garlic), and *A. schoenoprasum* (chives).

These and other *Allium* species have been investigated at molecular and DNA levels in an attempt to place them in proper taxa. The objective of this monograph is to present a survey of recent biochemical, molecular, and cytogenetic approaches that have been used on to elucidate genome organization in *Allium*. The intention is not to provide an exhaustive treatment of classification (for this refer to others [109, 31]), but rather to demonstrate the methods that have been used, singly and in combination, to understand the organization of the *Allium* genome.

## SPECIES HYBRIDIZATION

Hybridization plays a significant role in the production of novel phenotypes for the evolution and speciation of plants [126]. Crosses between *Allium* taxa have been made to attain novel types and to introgress desirable characters from one species into another. Interspecific hybridization within the genus has been of keen interest for many decades. The earliest of the crosses was between *Allium cepa* and *A. fistulosum* [24, 25, 66, 73]. Due to sterility barriers *A. cepa* x *A. fistulosum* interspecific derivatives are difficult to obtain [7, 15, 16, 24, 25, 67, 73, 84, 85, 86, 116], yet introgressants have been recovered in advanced *A. cepa* x *A. fistulosum* backcross populations [88].

Non-Mendelian inheritance of genes in interspecific hybrids and derivatives, manifested by full or partial sterility, results from pre- or postzygotic barriers. Various strategies to expose these barriers include

biochemical, cytogenetic, and DNA assays. In *Allium*, early mechanisms hypothesized that sterility of  $F_1$  generations and preferential genome transmission in advanced generations included stylar-incongruity [118] and nuclear-cytoplasmic incompatibility [7, 115, 117]. Pre-zygotic barriers observed in crosses of *A. cepa* x *A. ampeloprasum* [89] and *A. cepa* x *A. sphaerocephalon* [52] have been overcome with judicious breeding efforts. In *A. cepa* x *A. fistulosum*  $F_2$  and backcrosses, the nuclear genome most closely related to the maternal cytoplasmic genome is preferentially recovered in advanced generations [16, 116]; with persistent crossing, recombinants with paternal and maternal genomes have been recovered [41, 88]. Post-zygotic barriers selectively eliminate zygotes in plants causing distorted segregation in progeny populations. This distortion has been attributed to disharmony in interaction between parental genomes in the embryo and the endosperm [35]. A recent systematic examination of interspecific derivatives by Mangum and Peffley [74] describes a third mechanism which more fully explains distorted gene segregation and fecundity of advanced generations of *A. cepa* x *A. fistulosum* interspecific hybrids, i.e. central cell nuclear-cytoplasmic incongruity. The latter provides evidence for the underlying mechanism of incongruity in the central cell and predicts the success realized when genomes used for 'bridging' can be attributed to the resulting balanced and healthy endosperm formation. A success story using a bridge cross involves three genomes, *A. roylei*, *A. fistulosum* and *A. cepa*. Early studies reported these species to be closely related [64, 121] and that crosses between *A. fistulosum* and *A. roylei* resulted in a large extent of recombination [54]. Khrustaleva and Kik [55] used the *A. roylei* genome as a bridge between *A. fistulosum* and *A. cepa*. They observed that recovery of individuals with three genomes was possible because *A. roylei* genes could circumvent or restore the nucleo-cytoplasmic imbalance that led to the sterility shown by the (*A. fistulosum* x *A. cepa*) x *A. cepa* backcross of Ulloa et al. [117]. Information attained by using the underlying mechanisms was utilized using classical cytogenetic analyses of pure and derived (i.e. of species hybridization [97]) karyotypes and genomic hybridization, revealing recombinant chromosomes. This investigation exemplifies the current state of *Allium* research where progressively a synergistic blending of techniques as genomes is probed.

## BIOCHEMICAL AND MOLECULAR APPROACHES FOR CLASSIFICATION

The following section is a survey of salient molecular, cytogenetic, and chemotaxonomic approaches used in systematics of different taxa of Alliaceae. The literature is sorted according to the marker system used, listed alphabetically by taxa, followed by reference number. Where more than one approach has been used, the reference may be repeated or categorized by the most relevant approach; where more than one taxa has been investigated, the references may either be repeated, or sorted by taxa first appearing in the title.

Molecular markers have been used to elucidate geographic distribution patterns, patterns of genetic diversity, track introgression, identify hybrids, sort taxa, and detect maternal or paternal ancestry, construct genetic maps, genome analyses [61, 62], and in comparative studies [11]. Nuclear markers, which may be codominant (isozyme and microsatellites) or dominant [RAPDs (random amplified polymorphic DNA markers), RFLPs (restriction fragment length polymorphism), AFLPs (amplified fragment length polymorphism), and ITSs (internal transcribed spacers of nuclear ribosomal DNA)] are useful to track paternal or maternal inheritance. Mitochondrial markers have been associated with cytoplasmic male sterility (CMS) and used to distinguish male fertile from male sterile cytoplasm. Genomic changes may be revealed by cytogenetic chromosomal analyses and changes in nuclear repetitive DNA further elaborated with GISH (genomic in situ hybridization).

## DNA CONTENT IN *ALLIUM*

Among vascular plants there is approximately a hundred-fold variation in nuclear DNA content [10, 94]. *Alliums* are one of the several monocotyledonous plants (Table 1). Cultivated onion has nuclear DNA which is 40 times greater than that of rice, and in the case of lack it is 60 times greater. (0.8pg/2C) [4].

Nuclear DNA amounts of *Allium* vary widely by species [94] and are as low as 15.2 pg/2C (*A. sibiricum*) [49] and as great as 94.96 pg (picogram) in the octaploid *A. nutans* [6]. In a comparative study of nuclear DNA content of cultivated plant species across families, members of the Liliaceae and Alliaceae families have some of the largest genomes, *Tulipa*, *Allium ampeloprasum* and *A. cepa*, 63.6, 50.27, and

**Table 1.** Nuclear DNA content of some important monocotyledonous plant species determined by flow cytometry [from 4]

Scientific Name	Common Name	Family	Nuclear DNA Content	
			pg/2C <sup>a</sup>	mbp <sup>b</sup> /1C
<i>Aegilops squarrosa</i>	Goatgrass	Poaceae	8.34	4,024
<i>Allium ampeloprasum</i>	Leek	Alliaceae	50.27	24,255
<i>Allium cepa</i>	Onion	Alliaceae	32.74	15,797
<i>Asparagus officinalis</i>	Asparagus	Liliaceae	2.71	1,308
<i>Avena sativa</i>	Oats	Poaceae	23.45	11,315
<i>Hordeum vulgare</i>	Barley	Poaceae	10.10	4,873
<i>Oryza longistaminata</i>	African rice	Poaceae	0.78	376
<i>Oryza sativa ssp. Indica</i>	Rice	Poaceae	0.87-0.96	419-463
<i>Oryza sativa ssp. Japonica</i>	Rice	Poaceae	0.86-0.91	415-439
<i>Oryza sativa ssp. Javanica</i>	Rice	Poaceae	0.88	424
<i>Triticum aestivum</i> (2n=6X)	Wheat	Poaceae	33.09	15,966
<i>Triticum monococcum</i>	Einkorn wheat	Poaceae	11.92	5,751
<i>Tulipa</i> sp.	Garden tulip	Liliaceae	51.2-63.6	24,704-30,687
<i>Zea mays</i>	Corn	Poaceae	4.75-5.63	2,292-2,716

<sup>a</sup>Value for each cultivar determined by two or more measurements of at least 2,000 nuclei.

<sup>b</sup>1 picogram (pg) = 965 million base pairs (Mbp) (Bennett and Smith 1976)

32.74 pg/2C, respectively; diploid onion (*A. cepa*) carries the same amount as hexaploid wheat (*Triticum aestivum*) (33.09 pg/2C) [4, 75]. Accurate estimations of nuclear genomes are critical for plant genome studies. Precise quantification of DNA will aid in detecting nuclear DNA that has been modified by ploidy changes, introgressed segments, or additions or deletions of chromosomes or chromosome segments [95]. Establishing a plant standard in calculating the DNA content of plants may reduce a source of error, which affects estimations of DNA [48]. Johnston et al. [48] found general congruence of DNA contents when estimates as determined by flow cytometry were compared with those of standard Feulgen microspectrophotometry and a suggestion by them to launch an international, coordinated effort to set an agreed-upon plant calibration standard may lead to an agreement within the *Allium* community of researchers on baseline quantities of DNA.

Over 85% of the total genomic DNA in cereals consists of repetitive sequences, whereas genes constitute about 1%. Repetitive DNA

sequences represent the most variable part of the eukaryotic genome and can have major effects on genome organization and function [11]. Earlier cytological techniques such as C-banding [5, 50] and giemsa banding [87, 100] have been supplanted with more refined and precise cellular and molecular analyses, and gene localization *in situ* at the chromosomal level has greatly influenced the study of biodiversity. *In situ* hybridization in *Allium* is a powerful and accurate tool when mapping DNA onto chromosomes, including, but not limited to single copy [8, 19], or highly repetitive DNA sequences [65], satellite DNA [8], chromosomes/chromosome segments flow-sorted or microdissection [33], single gene location [96], total genomic DNA which provides a general picture of genomic divergence within taxa [99, 101], and alien chromosome segments [41]. Genomic *in situ* hybridization with total genomic DNA as a probe provides unique information about similarities between repetitive DNA from related species, as well as the physical location of conserved sequences on chromosomes; this method can yield a generalized picture of genomic divergence inside taxonomic groups. Telomeric repeat sequences (or lack of) can even more precisely predict phylogenetic groupings [2]. Molecular documentation serves as a useful aid in tracing genetic diversity and phylogenetic studies [61].

## MOLECULAR MARKER SYSTEMS

### Isozyme Analysis

*Allium cepa* [41, 70, 73, 77, 84, 98]

*Allium fistulosum* [42, 70, 77, 84, 88]

*Allium oschaninii* [70]

*Allium sativum* [44, 71]

*Allium vavilovii* [72]

*Allium* subg. *Allium* [61]

*Allium* subg. *Amerallium* [61]

*Allium* subg. *Bromatorrhiza* [61]

*Allium* subg. *Caloscordum* [61]

*Allium* subg. *Melanocrommyum* [61]

*Allium* subg. *Rhizirideum* [61]



## DNA Analysis

- i. Restriction fragment length polymorphism (RFLP) of chloroplast (cp) DNA

*Allium altaicum* [30]

*Allium cepa* var. *ascalonicum* [3]

*Allium fistulosum* [30]

*Allium* subg. *Melanocrommyum* [82]

*Allium* x *wakegi* [3]

*Allium* (29) spp. [78]

- mitochondrial (mt) DNA

*Allium ampeloprasum* [27, 38]

*Allium cepa* [26, 99]

*Allium commutatum* [57]

*Allium porrum* [57]

*Allium schoenoprasum* [27]

- genomic DNA

*Allium cepa* [60]

*Allium* subg. *Allium* [61]

*Allium* subg. *Amerallium* [61]

*Allium* subg. *Bromatorrhiza* [61]

*Allium* subg. *Caloscordum* [61]

*Allium* subg. *Melanocrommyum* [61]

*Allium* subg. *Rhizirideum* [61]

- ITS sequence analyses

*Allium* L. subg. *Melanocrommyum* [22, 23, 82]

Order Asparagales [63]

- Nuclear DNA

*Allium roylei* [121, 122]

- cDNA

*Allium cepa* [79]

- ii. Random amplified polymorphic DNA (RAPD)

*Allium aaseae* [107]

*Allium altaicum* [30]

*Allium ampeloprasum* [89]

*Allium cepa* [18, 60, 89, 112]

*Allium cepa* var. *ascalonicum* [3]

*Allium fistulosum* [30]

*Allium kermesinum* Rchb. [112]

*Allium oschaninii* [62]

*Allium sativum* [44, 70, 127]

*Allium* x *wakegi* [3]

*Allium vavilovii* [62]

*Allium* subg. *Allium* [61]

*Allium* subg. *Amerallium* [61]

*Allium* subg. *Bromatorrhiza* [61]

*Allium* subg. *Caloscordum* [61]

*Allium* subg. *Melanocrommyum* [29, 61]

*Allium* subg. *Rhizirideum* [61]

iii. Amplified fragment length polymorphism (AFLP)

*Allium cepa* [119, 120]

*Allium fistulosum* [120]

*Allium porrum* [105]

*Allium* subg. *Rhizirideum* [122]

*Allium roylei* [119, 120]

*Allium sativum* [44, 45]

iv. PCR-based markers

CMS

*Allium cepa* [26, 89, 90]

*Allium* section *cepa* [68]

*Allium ampeloprasum* [39, 90]

Anthocyanidin synthase

*Allium cepa* [59]

RT-PCR

*Allium cepa* [58]

CAPs (nrDNA)

*Allium* L. subg. *Melanocrommyum* (Webb et Berth.) Rouy [22]

*Allium giganteum* Regel. [21]

Indels

*Allium cepa* [47]

## SCAR

*Allium cepa* (shallot) [77]

*Allium fistulosum* [77]

## SNPs

*Allium cepa* [47, 76]

*Allium cepa* (shallot) [77]

*Allium sativum* [127]

## SSRs

*Allium cepa* [47, 76]

*Allium sativum* [127]

## v. Repetitive DNA and microsatellites

*Allium fistulosum* [46, 108]

## vi. Expressed sequence tags (ESTs)

*Allium cepa* [76, 79]

## vii. Telomeric repeats

Order Asparagales, Alliaceae [2]

*Allium altaicum* [17]

*Allium cepa* [2, 17, 32, 91]

*Allium chevsuricum* [32]

*Allium fistulosum* [91]

*Allium x proliferum* (Moench) Schrad. [91]

Alliaceae family genera *Allium*, *Nothoscordum*, *Tulbaghia* [1]

*Allium globosum* [32]

*Allium sativum* [32]

**Cytogenetic Chromosomal Analyses**

## i. In situ hybridization (ISH)

*Allium cepa* [41]

*Allium fistulosum* [41, 46]

## a. Fluorescence in situ hybridization (FISH)

*Allium altaicum* [17]

*Allium cepa* [17, 32, 56, 91, 102]

*Allium chevsuricum* [32]

*Allium fistulosum* [32, 91]

*Allium globosum* [32]

*Allium x proliferum* (Moench) Schrad. [91, 102]

*Allium schoenoprasum* [103]

*Allium sativum* [32]

Alliaceae family genera *Allium*, *Nothoscordum*, *Tulbaghia* [1]

b. Genomic in situ hybridization (GISH)

*Allium ampeloprasum* [90]

*Allium cepa* [9, 62, 90, 101, 110]

*Allium cepa* var. *viviparum* [92]

*Allium fistulosum* [9, 110]

*Allium oschaninii* [31, 62]

*Allium vavilovii* [31, 62]

*Allium wakegi* [103]

*Allium* subg. *Melanocrommyum* [29]

ii. Chromosomal analyses

a. Bromodeoxyuridine (BrdU)

*Allium fistulosum* [34]

b. C-banding

*Allium altynolicum* [28]

*Allium fistulosum* [46]

c. Flow cytometry/karyotype

*Allium albidum* Fisch. ex Bieb. [52]

*Allium angulosum* [52]

*Allium carolinianum* DC [52]

*Allium cepa* [52]

*Allium chevsuricum* Tscholok. [52]

*Allium flavellum* Vved [52]

*Allium hymenorrhizum* Ldb. [52]

*Allium jodanthum* Vved. [52]

*Allium karelinii* Poljak. [52]

*Allium lineare* L.s.l. [52]

*Allium obliquum* L. [52]

*Allium rubens* Schad. ex Willd. [52]

*Allium saxatile* M. Bieb. [52]

*Allium sphaerocephalon* [52]

*Allium victorialis* L. [52]

## CHEMOTAXONOMIC RELATIONSHIPS

Health benefits of *Allium* species for their phytochemical value and application as phytopharmaceuticals is gaining significance [39, 40, 43]. Recent advances in chemotaxonomic relationships have identified constituents unique to species [13, 14]. Application of chemotaxonomy in crop improvement allows for selection of germplasm to design novel phenotypes with desired phytochemical constituency. Aroma profiles in *Allium* have been found to be taxa specific [12] and, thus, are useful for chemotaxonomical classification [111]. Sulphur-containing compounds responsible for the characteristic onion aroma and classes of flavonoids responsible for scale and leaf pigmentation are mainly accountable for the health properties found in *Alliums*. As more information is gained about these classes of compounds, they become candidates for single gene insertion and targets for manipulation [58]. The major flavonol found in onion (*Allium cepa*), quercetin, could become a selection trait in breeding programs because of: (1) potential benefits to human health, (2) it is largely unaffected by the environment [69, 83], and (3) is genetically inherited [106].

### Chemical Constituents

- i. Aroma, sulfur-containing compounds, cystein sulfoxides (CS)  
*Allium* seven chemotypes of 43 species [110]  
*Allium cepa* [53, 112]  
*Allium globosum* [53]  
*Allium kermesinum* Rchb. [112]  
*Allium obliquum* [53]
- ii. Flavonoids separated into groups by detection of flavonols and flavones  
*Allium cepa* *Aggregatum* Group [104]  
*Allium fistulosum* [104]  
*Allium* Section *Allium* (37 species) [37]  
*Allium Ampeloprasum* Group [37]  
*Allium Guttatum* Group [37]  
*Allium Rotundum* Group [37]  
*Allium Sphaerocephalon* Group [37]
- iii. Anthocyanidin synthase  
*Allium cepa* [58]

## CONCLUSION

Given its wide variability, *Allium* continues to be an interesting crop, at cytogenetic, chemical, and molecular levels. As it has one of the largest genomes in the plant kingdom and is taxonomically complex, it poses unique challenges and opportunities for elucidating genome organization. Significant progress has been made in recent years in revealing genomic organization at the chromosomal and DNA levels. The emerging technology of proteomics may provide even greater insight into the genomes and possibilities of protein manipulation. Marker-assisted breeding is routine in many *Allium* improvement programs and with the wide array of target species and plethora of investigative methodologies, great strides are being made towards better understanding of this complex genus.

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# Molecular Phylogenetics of Chinese *Cymbidium* (Orchidaceae) Based on nrITS Sequence and RAPD Data

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## ABSTRACT

The sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA nr(DNA) and random amplified polymorphic DNA (RAPD) data were used to evaluate the genetic diversity and phylogenetic relationships of *Cymbidium* of China. Variant of ITS sequence is low among genus *Cymbidium* and could only partly define the phylogenetic relationships of *Cymbidium* although van den Berg et al. [21] found the sequence variant levels of *Cymbidium* to be higher than other orchids. The phylogenetic tree generated from ITS sequences is only partially congruent with the current taxonomic classification of the genus at the subgenus level, but better congruent with the traditional section classification, except *C. dayanum*, which was placed with the member subsection *Cyperorchis*. The removal of *C. dayanum*, subgenus *Cyperorchis* was a monophyletic group; and subgenus *Jensoa* also appeared paraphyletic, with *C. lancifolium* being the sister-group to the remaining genus; species of subgenus *Cymbidium* appeared polyphyletic, being split into several clades and intermixed with the main subgenus *Cyperorchis* and subgenus *Jensoa* clades, respectively. The unweighted pair-group methods with arithmetical averages (UPGMA) dendrogram based on RAPD data further confirmed that *C. dayanum*

was located in subgenus *Cyperorchis*, and high polyphyly of subgenus *Cymbidium*. However, some apparent incongruence could be noted between the phylogenetic relationships constructed from the ITS and RAPD data. The RAPD data also showed that the cultivars of *C. goeringii* and *C. eburneum* had higher genetic diversity than other species, which could have originated from the long history of cultivation and selection, and geographic isolation.

**Key Words:** *Cymbidium*, Orchidaceae, phylogeny, ITS, RAPD

**Abbreviations:** nrITS = Internal transcribed spacers of nuclear ribosomal DNA RAPD = Random amplified polymorphic DNA.

## INTRODUCTION—DISTRIBUTION PATTERNS

*Cymbidium* is one genus of the family Orchidaceae, which is of immense importance in horticulture. There are about 48 species of *Cymbidium* (Orchidaceae) in the world, and about 29 species and varieties in China [5, 6]. It is widely distributed in southeast Asia, from northwest India to Japan, and south Australia, with the species diversity center in northeast India, southwest China, Indo-China and Malaysia. *Cymbidium* is most abundant in southwest China, followed by the southeast China, especially in Yunnan Province, where this species flourishes [23] (Table 1).

*C. goeringii* Rchb.f. and *C. faberi* Rolfe are most widely distributed, which often grow in the cold areas such as south of Ganshu Province, south of Qingling mountain of Shanxi Province, Heinan, Anhui, Hubei, Hunan, Jiangxi, Zhejiang, Jiangshu, Taiwan, Fujian, Guangxi, Sichuan, Guizhou, Yunnan, Xizhuang Province, and northeast of Guangdong Province. *C. kanran* Makio., *C. floribundum* Lindl., and *C. lancifolium* Hook. are found in south China, including Hubei, Hunan, Jiangxi, Fujian, Zhejiang, Taiwan, Guangdong, Guangxi, Sichuan, Guizhou, and Yunnan. *C. ensifolium* (L.) Sw. is distributed in south of Zhejiang, Jiangxi, Fujian, Taiwan, Hunan, Guangdong, Guangxi, Hainan, Sichuan, Guizhou, and Yunnan Province; *C. sinense* Jackson ex Andr. Willd. in Taiwan, Fujian, south of Sichuan, Jiangxi, Anhui, Guangdong, Hainan, Guangxi, Guizhou, and Yunnan. *C. hookerianum* Rchb.f., *C. iridioides* D.Don and *C. tracyanum* L.Castle are found in Sichuan, Guizhou, Yunnan, Xizhang, and Guangxi Province. *C. insigne* Rolfe only is found in Hainan Province [21].

Table 1. The distribution of *Cymbidium* in provinces of China

Species	Distribution in provinces of China														Altitude above sea level ('M')
	SX	He	GS	AH	HB	Hu	ZJ	JX	JS	TW	FJ	GX	GD	Ha	GZ
<i>C. aloifolium</i>											✓	✓	✓		✓
<i>C. bicolor</i>												✓	✓	✓	✓
<i>C. dayanum</i>										✓	✓	✓	✓	✓	✓
<i>C. floribundum</i>					✓	✓	✓	✓							✓
<i>C. Suawissimum</i>															✓
<i>C. tracyanum</i>															✓
<i>C. iridioides</i>														✓	✓
<i>C. erythraeum</i>														✓	✓
<i>C. hookerianum</i>												✓			✓
<i>C. wilsonii</i>															✓
<i>C. lowianum</i>															✓
<i>C. insigne</i>														✓	✓
<i>C. Wenshanense</i>															✓
<i>C. eburneum</i>												✓		✓	unclear
<i>C. mastersii</i>															✓
<i>C. elegans</i>														✓	✓
<i>C. cochleare</i>										✓	✓				✓
<i>C. tigrinum</i>															✓
<i>C. ensifolium</i>				✓			✓	✓		✓	✓	✓	✓	✓	✓

Table 1 contd.



Table 1 contd.

<i>C. sinense</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	300-2,000
<i>C. defoliatum</i>									✓	✓	✓	
<i>C. nanulum</i>								✓			✓	unclear
<i>C. kanran</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	400-2,400
<i>C. cyperifolium</i>							✓	✓	✓	✓	✓	900-1,600
<i>C. faberi</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	700-3,000
<i>C. Goeringii</i>	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	300-2,200
<i>C. qubeinse</i>											✓	700-1,800
<i>C. lancifolium</i>			✓	✓	✓	✓	✓	✓		✓	✓	300-2,200
<i>C. macrorrhizon</i>										✓	✓	700-1,500

SX: Shanxi; He: Henan; GS: Ganshu; AH: Anhui; HB: Hubei; Hu: Hunan; ZJ: Zhejiang ; JX: Jiangxi; JS: Jiangshu; TW: Taiwan; FJ: Fujian;  
GX: Guangxi; GD: Guangdong; Ha: Hainan; SC: Sichuan; YN: Yunnan; XZ: Xizhang; GZ: Guizhou

*Cymbidium* species usually occur in the zone of 100-2,000 m above sea level. Looking from the growing latitude, the *Cymbidium* distribute at the north latitude  $24^{\circ}$ - $34^{\circ}$ . From the uprightness, different areas yield the same *Cymbidium* species but different sea levels and different *Cymbidium* species can grow on the same altitude. Two to three species of *Cymbidium* can grow in the same sea level, for instance, *C. Goeringii* and *C. sinense* often grow together (Fig. 1).

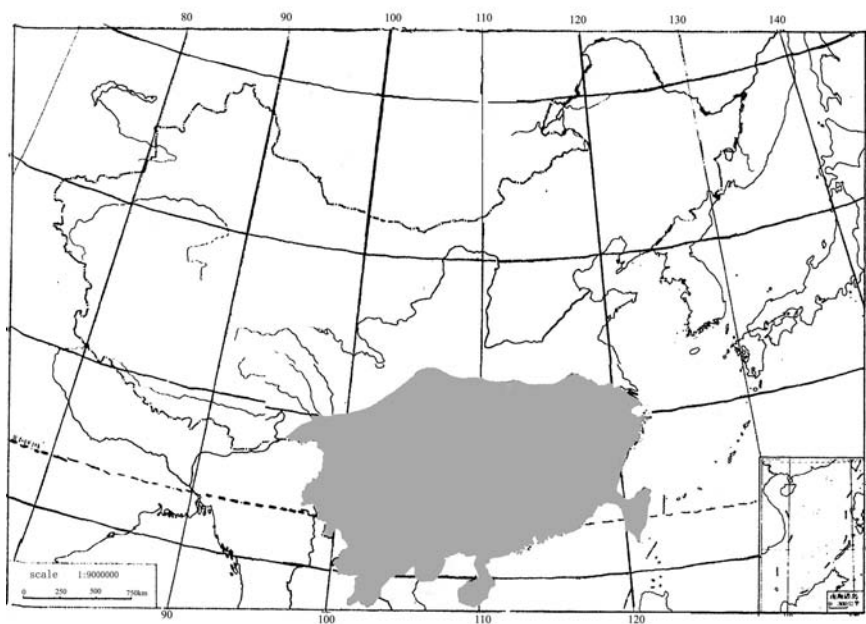


Fig. 1. The distribution map of *Cymbidium* in China. *Cymbidium* grows in the shadow zone of China.

## THE TRADITIONAL TAXONOMY IN *CYMBIDIUM*

Du Puy and Cribb [9] have reviewed four main classifications of the genus *Cymbidium* (Table 2). In Dressler's [8] framework of Orchidaceae, *Cymbidium* is placed in Cymbidieae of Vandioideae, which contains all the sympodial vandoid orchids mostly with two pollinia. The generic delimitation of *Cymbidium* has been controversial, largely due to the different emphasis of the character variation of pollinia and lip (Table 2). Schlechter [15] completed revision of this group of plants on the basis of the modern infrageneric classification of *Cymbidium* although two genera, *Cymbidium* and *Cyperorchis*, were recognized in his system. He emphasized the fusion of the base of lip and the base of column as the

**Table 2.** Comparison of traditional supraspecific classifications proposed for *Cymbidium* (modified from Du Puy and Cribb [9])

Blume	Schlechter	Seth & Cribb	Du Puy & Cribb
<i>Cymbidium</i> Sw.	<i>Cymbidium</i> Sw.	<i>Cymbidium</i> subgen. <i>Cymbidium</i>	<i>Cymbidium</i> subgen. <i>Cymbidium</i>
	sect. <i>Eucymbidium</i>	sect. <i>Cymbidium</i>	sect. <i>Cymbidium</i>
	sect. <i>Himantophyllum</i>	sect. <i>Himantophyllum</i>	sect. <i>Borneense</i>
	sect. <i>Austrocymbidium</i>	sect. <i>Austrocymbidium</i>	sect. <i>Himantophyllum</i>
<i>Iridorchis</i> Bl.	sect. <i>Bigibbarium</i>	sect. <i>Floribundum</i>	sect. <i>Austrocymbidium</i>
	<i>Cyperorchis</i> Bl.	sect. <i>Suavissimum</i>	sect. <i>Floribundum</i>
	sect. <i>Iridorchis</i>	sect. <i>Bigibbarium</i>	sect. <i>Bigibbarium</i>
		subgen. <i>Cyperorchis</i>	subgen. <i>Cyperorchis</i>
<i>Cyperorchis</i> Bl.	sect. <i>Annamaea</i>	sect. <i>Iridorchis</i>	sect. <i>Iridorchis</i>
	sect. <i>Eucyperorchis</i>	sect. <i>Eburnea</i>	sect. <i>Eburnea</i>
	sect. <i>Parishiella</i>	sect. <i>Annamaea</i>	sect. <i>Annamaea</i>
	<i>Cymbidium</i> Sw.	sect. <i>Cyperorchis</i>	sect. <i>Cyperorchis</i>
	sect. <i>Jensoa</i>	sect. <i>Parishiella</i>	sect. <i>Parishiella</i>
	sect. <i>Maxillarianthe</i>	subgen. <i>Jensoa</i>	subgen. <i>Jensoa</i>
	sect. <i>Geocymbidium</i>	sect. <i>Jensoa</i>	sect. <i>Jensoa</i>
	sect. <i>Macrorhizon</i>	sect. <i>Maxillarianthe</i>	sect. <i>Maxillarianthe</i>
		sect. <i>Geocymbidium</i>	sect. <i>Geocymbidium</i>
		sect. <i>Pachyrrhizanthe</i>	sect. <i>Pachyrrhizanthe</i>

unique distinguishing feature of *Cyperorchis*, rather than emphasizing the pollinarium and pollinium shapes as did Blume [2, 3, 4], Reichenbach [13] and Hooker [10]. Schlechter [15] also proposed several sections for both genera. Hunt [11] reduced *Cyperorchis* to within *Cymbidium* and maintained Schlechter's sectional divisions. Seth and Cribb [16] started to use the subgenus concept and three subgenera were proposed, mainly based on the number of pollinia and state of fusion between lip and column: subgenus *Cymbidium* with two pollinia and free lip, subgenus *Cyperorchis* with two pollinia and fusion of lip and column-base, and subgenus *Jensoa* with four pollinia and free lip. This system was slightly modified by Du Puy and Cribb [9].

In this chapter analysis of phylogenetic relationship in *Cymbidium* of China has been undertaken based on the sequences of the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (nrDNA) and RAPD. ITS sequences [1, 21, 27] and RAPD [12, 25, 26] have been widely used to infer phylogenetic relationships among closely related genera and species. RAPD plays an important role in the research of genetic diversity and the relationships of species. The phylogenetic relationships formulated were evaluated against the existing supraspecific classification proposed by Du Puy and Cribb [9].

## **ANALYSIS OF PHYLOGENETIC RELATIONSHIPS IN CYMBIDIUM (ORCHIDACEAE) BASED ON nrITS SEQUENCE DATA**

A total of 30 taxa of *Cymbidium* from around China were sampled (voucher specimens deposited in South China Botanical Garden (SCBG)). Their distribution, source, and the GenBank accession numbers are shown in Table 3. Du Puy and Cribb's [9] classification of *Cymbidium* was followed for the purpose of this phylogenetic analysis. Two species from tribe Cymbidieae, *Eulophia graminea*, *Geodorum densiflorum*, and one from tribe Orchideae, *Amitostigma pinguiculum*, were designated as outgroups. All samples for ITS were collected from the wild in China and cultivated in SCBG.

Total DNA was extracted from fresh leaves, following the 2 x CTAB protocol [7]. Nuclear ribosomal internal transcribed spacers (ITS1, 5.8S, ITS2 region) were amplified using the forward primer ITS5 [22] and the reverse primer CA26 [24]. Amplified double stranded DNA fragments were purified using QIAquick Gel Extraction Kit (QIAGEN). Automated sequencing was performed using fluorescent dye-labeled

**Table 3.** The species and cultivars of *Cymbidium* in this study

No. of sample	Species or cultivars	Source; voucher specimen	GenBank accession number
(1)	<i>C. aloifolium</i> (L.) Sw	Yunnan; Sun 98-2	AF284695
(2)	<i>C. aloifolium</i> (L.) Sw 'xishanwenbanlan'	Yunnan; Sun 98-1	
(3)	<i>C. aloifolium</i> (L.) Sw 'xiaguanwenbanlan'	Yunnan; Sun 98-3	
(4)	<i>C. bicolor</i> Lindl.subsp. <i>obtusum</i> Du Puyet Cribb	Yunnan; Sun 98-4	AF284696
(5)	<i>C. dayanum</i> Rchb.f.	Yunnan; Sun 98-5	AF284697
(6)	<i>C. floribundum</i> Lindl.	Yunnan; Sun 98-6	AF284698
(7)	<i>C. floribundum</i> Lindl. 'tailanta'	Yunnan; Sun 98-10	
	<i>C. pumilum</i> Rolfe	Yunan; Sun 98-7	AF284699
(8)	<i>C. suavissimum</i> Sander ex C.Curtis	Yunnan; Sun 98-8	AF284700
(9)	<i>C. tracyanum</i> L. Castle	Yunnan; Sun 98-9	AF2284701
(10)	<i>C. hookerianum</i> Rchb.f.	Yunnan; Sun 98-51	AF284702
(11)	<i>C. wilsonii</i> (Rolfe ex Cook) Rolfe	Yunnan; Sun 98-11	AF284703
(12)	<i>C. insignie</i> Rolfe	Zhejiang; Sun 98-12	AF284704
(13)	<i>C. iridioides</i> D. Don	Yunnan; Sun 98-13	AF284705
(14)	<i>C. erythraeum</i> Lindl.	Yunnan; Sun 98-14	AF284706
(15)	<i>C. lowianum</i> (Rchb.f) Rchb.f.	Yunnan; Ye 15	AF284707
(16)	<i>C. wenshanense</i> Y.S.Wu et F.Y. Liu	Yunnan; Sun 98-16	AF284708
(17)	<i>C. elegans</i> Lindl.	Yunnan; Sun 98-19	AF284709
(18)	<i>C. mastersii</i> Griff. ex Lindl.	Yunnan; Sun 98-21	AF284710
(19)	<i>C. mastersii</i> Griff. ex Lindl. 'maguanlan'	Yunnan; Sun 98-18	

Table 1 contd.

Table 1 contd.

(20)	<i>C. eburneum</i> Lindl.	Yunnan; Sun 98-22	AF284711
(21)	<i>C. eburneum</i> Lindl. 'xiangyabai'	Hainan; Sun 98-32	AF284712
(22)	<i>C. tigrinum</i> Parish ex Hook.	Yunnan; Sun 98-31	AF284713
	<i>C. ensifolium</i> (L.) Sw.	Guangdong; Ye 32	AF284714
(23)	<i>C. ensifolium</i> (L.) Sw. 'shaoguanjianlan'	Guangdong; Ye 31	AF284715
(24)	<i>C. ensifolium</i> (L.) Sw. 'daqing'	Guangdong; Ye 33	
(25)	<i>C. ensifolium</i> (L.) Sw. 'tiegushu'	Yuannan; Sun 98-35	
(26)	<i>C. sinense</i> (Jackson ex Andr.) Willd.	Guangdong; Zhang 98-23	AF284716
(27)	<i>C. sinense</i> (Jackson ex Andr.) Willd. 'baimo'	Guangdong; Zhang 98-24	AF284717
(28)	<i>C. sinense</i> (Jackson ex Andr.) Willd. 'xianyimo'	Yunnan; Sun 98-36	
(29)	<i>C. sinense</i> (Jackson ex Andr.) Willd. 'hainanmo'	Hainan; Sun 98-37	
(30)	<i>C. sinense</i> (Jackson ex Andr.) Willd. 'heimo'	Guangdong; Sun 98-40	
(31)	<i>C. sinense</i> (Jackson ex Andr.) Willd. 'qihei'	Guangdong; Sun 98-41	
(32)	<i>C. sinense</i> (Jackson ex Andr.) Willd. 'ruanjianbaimo'	SCBG	
(33)	<i>C. defoliatum</i> Y.S. Wu et S.C. Chen.	Yunnan; Sun 98-33	AF284718
(34)	<i>C. cyperifolium</i> Wall.ex Lindl.	Yunnan; Sun 98-34	AF284719
(35)	<i>C. kanran</i> Makio	Guangdong; Ye 37	AF284720
(36)	<i>C. kanran</i> Makio 'qinghanlan'	Guangdong; Sun 98-42	
(37)	<i>C. kanran</i> Makio 'zihanlan'	Yunnan; Sun 98-44	
(38)	<i>C. faberi</i> Rolfe.	Yunnan; Sun 98-38	AF284721
(39)	<i>C. goeringii</i> (Rchb.f.) Rchb.f.	Yunnan; Sun 98-39	AF284722
(40)	<i>C. goeringii</i> (Rchb.f.) Rchb.f. 'duoxiang'	Yunnan; Sun 98-45	

Table 1 contd.

Table 1 contd.

(41)	<i>C. goeringii</i> (Rchb.f.) Rchb.f. 'doubanlan'	Yunnan; Sun 98-46	
(42)	<i>C. goeringii</i> (Rchb.f.) Rchb.f. var. <i>longibracteatum</i>	Yunnan; Sun 98-48	
(43)	<i>C. goeringii</i> (Rchb.f.) Rchb.f. 'bailhuacunjian'	Yunnan; Sun 98-49	
(44)	<i>C. goeringii</i> (Rchb.f.) Rchb.f. 'guangdongduoxiang'	Guangdong; Sun 98-50	
(45)	<i>C. Lianbanlan</i> Tang et Wang	Yunnan; Sun 98-47	AF284723
(46)	<i>C. Lianbanlan</i> Tang et Wang 'huanghualianbanlan'	Yunnan; Sun 98-51	
(47)	<i>C. lancifolium</i> Hook.	Yunnan; Sun 98-43	AF284724
(48)	<i>C. Sinense</i> x <i>C. ebumeum</i>	SCBG	
(49)	<i>C. tracyanum</i> x <i>C. ebumeum</i>	SCBG	
(50)	<i>C. x hybridus</i>	SCBG	
	<i>Eulophia graminea</i> Lindl	Yunnan; Sun 98-62	AF284726
	<i>Geodonum desiflorum</i> GeLamLa	Yunnan; Sun 98-63	AF284727
	<i>Amitostigma pinguicula</i> (Rchb.f. et S. Moore) Schltr	Yunnan; Sun 98-61	AF284725

The numbers in the bracket are No. of samples for RAPD, and samples for ITS are given the Gene-Bank accession number.

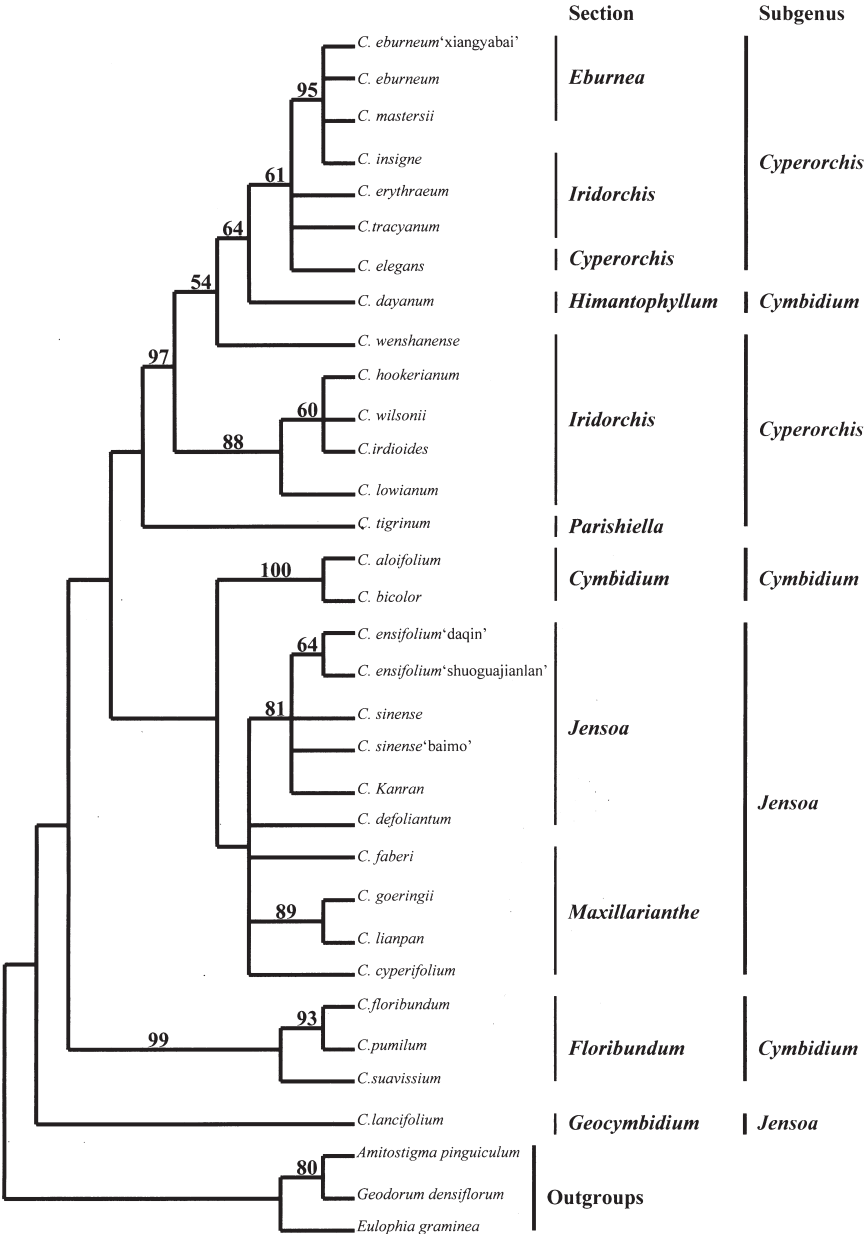
nucleotides on an ABI 377 DNA Sequencer, using at least two of the four primers of N18L18 [24], ITS2, ITS3 and ITS4 [22].

The combined sequences of ITS1, 5.8S and ITS2 were aligned using Clustal X [20], adjusted manually where necessary. Maximum parsimony analyses were performed using PAUP 4.0 [19] with all changes weighted equally, using HEURISTIC searches with TBR branch swapping and 100 random additional sequences. Multiple most parsimonious trees were summarized as strict consensus tree. To assess the relative support for clades found in the parsimony analysis, bootstrap analysis (BS) was conducted using 1,000 replicates and the same tree search procedure as described above, except with a simple taxon addition.

The length of the entire ITS region in the *Cymbidium* species surveyed ranged from 646 bp (base pairs) to 661 bp, with ITS1 ranging from 240 to 245 bp, 5.8 S 162 bp for all species, and ITS2 ranging from 243 to 255 bp. Of the 681 aligned ITS sequence, 132 bp sites were variable with 25 sites being potentially phylogenetically informative. There was one INDEL (insertion/deletion) of an 8-base pair (bp) deletion in the ITS2 region that was observed in subgenus *Cyperorchis* and *C. dayanum* of subgenus *Cymbidium*, but not in other *Cymbidium* species (aligned matrix not shown).

The maximum parsimony analysis, treating gaps as missing data, resulted in 33 most parsimonious trees, with a length of 272 steps,  $CI=0.66$  (excluding uninformative characters), and  $RI=0.79$ . These trees essentially differ in the internal composition of the clade comprising subgenus *C. yperochis* and *C. dayanum*, and the clade comprising subgenus *Jensoa* (without *C. lancifolium*). The strict consensus tree is presented in Fig. 2. There is consistent but weak support for a sister-group relationship between *C. lancifolium* and the clade containing all other *Cymbidium* species ( $BS < 50$ ). The latter consists of two clades, one including three species of subgenus *Cymbidium* section *Floriundum*, with high bootstrap support ( $BS = 99$ ). The other clade comprises two subclades (both with  $BS < 50$ ); one subclade consists of all species of subgenus *Cyperorchis* and *C. dayanum* of subgenus *Cymbidium* ( $BS < 50$ ), and the other includes two species of subgenus *Cymbidium* (*C. aloifolium* and *C. bicolor*) with 100% bootstrap support, which becomes the sister to the clade containing all species of subgenus *Jensoa* except *C. lancifolium* ( $BS < 50$ ). Poor resolution is presented for species relationships of both subgenus *Cyperochis* and subgenus *Jensoa*.





**Fig. 2.** Strict consensus of the 33 most parsimonious trees from the entire ITS sequences with gaps coded as missing. Tree length, 272 steps; CI-0.66; RI-0.79. Numbers above lines represent bootstrap values in 1,000 replicates. Supraspecific classification follows Du Puy and Cribb [9]. Figure was from [27].

Treating gaps as a fifth state, the maximum parsimony analysis generated 33 most parsimonious trees, with a length of 325 steps, a CI of 0.68 (excluding uninformative characters), and RI of 0.83. The strict consensus tree is largely congruent with that of treating gaps as missing data (Fig. 2), except that *C. erythraeum* becomes the sister to the clade comprising *C. eburneum* CV, *C. eburneum*, *C. mastersii*, and *C. insigne* (figure not shown).

Level of variability was low across all accessions of *Cymbidium* species in the ITS region studied, although van den Berg et al. [21] considered the variant of *Cymbidium* as higher than other orchids. The limited number of parsimony-informative characters resulted in relatively weak support for some of the clades identified (especially for the major lineages of *Cymbidium*) and several conclusions discussed below should be tested with additional data.

The ITS tree, however, shows that such subgenus delimitations [9] should be evaluated with more data. Subgenus *Cyperorchis* is not a monophyletic group, with the unexpected nesting of *C. dayanum* (subgenus *Cymbidium*) within it. However, with removal of the *C. dayanum* or considering it as a member of subgenus *Cyperorchis*, it would be a monophyletic group. *C. dayanum*, representing section *Himantophyllum*, is placed in subgenus *Cymbidium* with two cleft pollinia and without any fusion between the lip and base of the column. It is distinctive subgenus *Cymbidium* and superficially resembles some of the species in subgenus *Jensoa*, especially in the vegetative characteristics and the slender, acute, arching leaves [9]. The systematic position of *C. dayanum* clearly merits additional study.

*C. tigrinum*, the single, highly distinctive species representing section *Parishiella*, is sister to the remaining subgenus *Cyperorchis* and *C. dayanum*, although with low bootstrap support (<50%). It is quite different from other *Cymbidium* species, especially vegetative characteristics, but possesses all the diagnostic characters of subgenus *Cyperorchis*. It probably has close affinity with section *Iridorchis*, which the pollinarium shape closely resembles [9].

Subgenus *Jensoa* also appears paraphyletic in the ITS tree (Fig. 2), with *C. lancifolium* becoming the sister-group to the remaining of the genus, although with low bootstrap support (<50%). *Cymbidium lancifolium*, the single species comprising section *Geocymbidium*, is the most widespread species in the genus and highly distinctive in vegetative characteristics. Its habitat (or traits) and flowers indicate a close

relationship with the saprophytic species *C. macrorhizon* of section *Pachyrhizanth*e, which lacks leaves and chlorophyll, and is apparent only when in blossom [9]. It is speculated from the early divergence of *C. lancifolium* that ancestors of *Cymbidium* species diversified along two paths, one leading to the main epiphytic or lithophytic species, the other into the saprophytic ones. Apparently, the addition of the saprophytic species of section *Pachyrhizanth*e will be invaluable to verify this hypothesis of evolutionally diversifying paths.

Two species of section *Cymbidium*, *C. aloifolium* and *C. bicolor*, constitute the sister-group to subgenus *Jen*soa (without *C. lancifolium*). The splitting feature of subgenus *Cymbidium* apparent in the ITS tree suggests that subgenus *Cymbidium* is polyphyletic. The number of pollinia is usually considered to be a conservative character and has been employed for the infrageneric classification of *Cymbidium* [9]. Nevertheless, this criterion may not be general: the Borneo species *C. borneense* of section *Borneense* has four pollinia, but has more other diagnostic characters and is more reliably to be placed in subgenus *Cymbidium*. No matter whether the two or four pollinia are advanced, transformation of this character may have occurred more than once in *Cymbidium*.

In comparison to the results of van den Berg et al. [21], phylogenetic relationship of Chinese *Cymbidium* based on ITS sequence is similar: the tree generated from ITS sequences is only partially congruent with the current subgenus classification, although the section groups are better congruent with the traditional classification. Both ITS [21, 27] and *matK* sequences [21] have low variation which could not completely solve the phylogenetic relationship between each species of *Cymbidium*.

## **RELATIONSHIP BETWEEN SPECIES, CULTIVARS OF CYMBIDIUM BASED ON RAPD DATA**

Due to insufficiency of informative characters of ITS among most of *Cymbidium* species to solve their phylogenetic relationship, the more effective diversity molecular marker RAPD was further used to evaluate the relationships among *Cymbidium* species and some cultivars. A total of 50 samples (Table 3 comprising 28 original species, 19 cultivars among 8 species, and 3 hybrids) were used in this study, which were taken from Yunnan or Guangdong, and planted in South China Garden of Botany.

The total DNA extraction followed the 2xCTAB protocol [17] from fresh leaves, except dry leaf of *C. insign*e. Amplification of genomic DNA

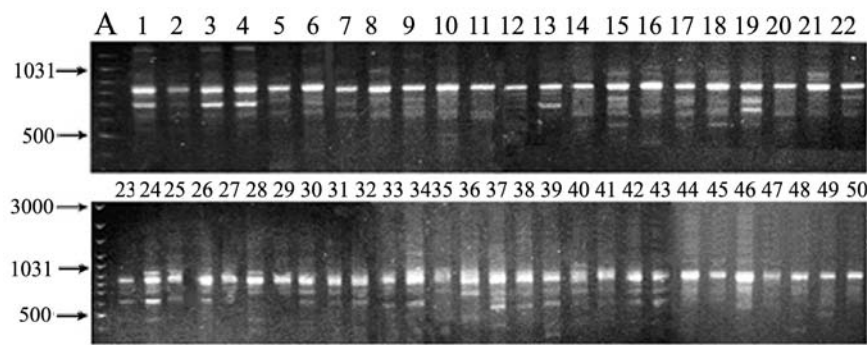
was made on a MJ DNA Cycler using the arbitrary decamers. The 12 oligoprimers from 100 RAPD primer kit (Sangon, Shanghai, China) were selected for conducting polymerase chain reaction (PCR) by pre-screening since the 12 primers could produce diversity bands (Table 4). Amplifications of genomic DNA were performed in 20- $\mu$ l reaction volumes containing 1.0 units of Taq DNA polymerase (Sino-US, China), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.3  $\mu$ M each of random primer and 40 ng of template DNA. The cycle program included an initial 3 minutes denaturation at 94°C, followed by 45 cycles of 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C, with a final extension at 72°C for 10 minutes. RAPD fragments were separated electrophoretically on 1.5% agarose gels in 1X TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator using a digital camera. DNA from each plant was amplified with the same primer more than once, and the banding patterns were compared.

**Table 4.** Sequence of the selected random nucleotide primers used in RAPD analysis

Primer	Sequence	Primer	Sequence
S106	ACGCATCGCA	S167	CAGCGACAAG
S121	ACGGATCCTG	S174	TGACGGCGGT
S133	GGCTGCAGAA	S178	TGCCCAGCCT
S140	GGTCTAGAGG	S180	AAAGTGCGGG
S142	GGTGCGGGAA	S198	CTGGCGAACT
S143	CCAGATGCAC	S199	GAGTCAGCAG

Fragment sizes were designated as amplified bands, and bands were shared as diallelic characters (present = 1, absent = 0). Those bands amplified in each instance were scored and included in the analyses. UPGMA [18] clades analysis were computed from Jaccard coefficient using NTSYS-pc V1.80 [14].

A total of 250 amplified bands was obtained from all PCR reactions by 12 selected primers, and their band size ranged from 200 bp to 2,100 bp. Except of the 850 bp band amplified from S143 primer, all other amplified bands were diversity locus, and most of the bands were located between 1,030 bp and 300 bp (Fig. 3).



**Fig. 3.** The RAPD amplified profile using the primer S143. No. of samples was indicated in Table 3.

Jaccard’s coefficient (JC) was mainly arranged from 0.100 to 0.250 each other among the 50 samples, The JC of *C. ensifolium* ‘daqin’ and *C. ensifolium* ‘tiegushu’ is biggest (0.644) and the JC of *C. goeringii* and *C. lowianum* is smallest (0.050) in all the JCs.

Except *C. eburneum* and *C. goeringii*, the cultivars of *C. aloifolium*, *C. mastersii*, *C. sinense*, *C. ensifolium*, *C. Kanran*, *C. lianpan* and *C. floribundum* were grouped together within their species, respectively, in the UPGMA dendrogram tree generated from the RAPD data (Fig. 4). These results showed that RAPD could be used to evaluate the relationship of species and their cultivars. Six cultivars of *C. goeringii* were divided into three groups and separated, and two cultivars of *C. eburneum* were also separated, which indicated more genetic diversity between these two species than others. From Table 3, two cultivars of *C. eburneum* were obtained from southwest China (Yunnan) and the southernmost island of China (Hainan island), and six cultivars of *C. goeringii* were from Yunnan and Guangdong (south China). These two species were separated by high mountains or strait. This geographic isolation may confer to the greater genetic diversity among the cultivars of *C. eburneum* and *C. goeringii* than other species.

The classic classification [9] put the Chinese *Cymbidium* into 3 subgenus and 11 sections. Subgenus *Cymbidium* was divided into the section of *Cymbidium*, *Himantophyllum* and *Floribundum*, Subgenus *Cyperorchis* into section of *Iridorchis*, *Cyperorchis*, *Eburnea* and *Parishiella*, Subgenus *Jensoa* into section of *Jensoa*, *Maxillarianthe*, *Geocymbidium* and

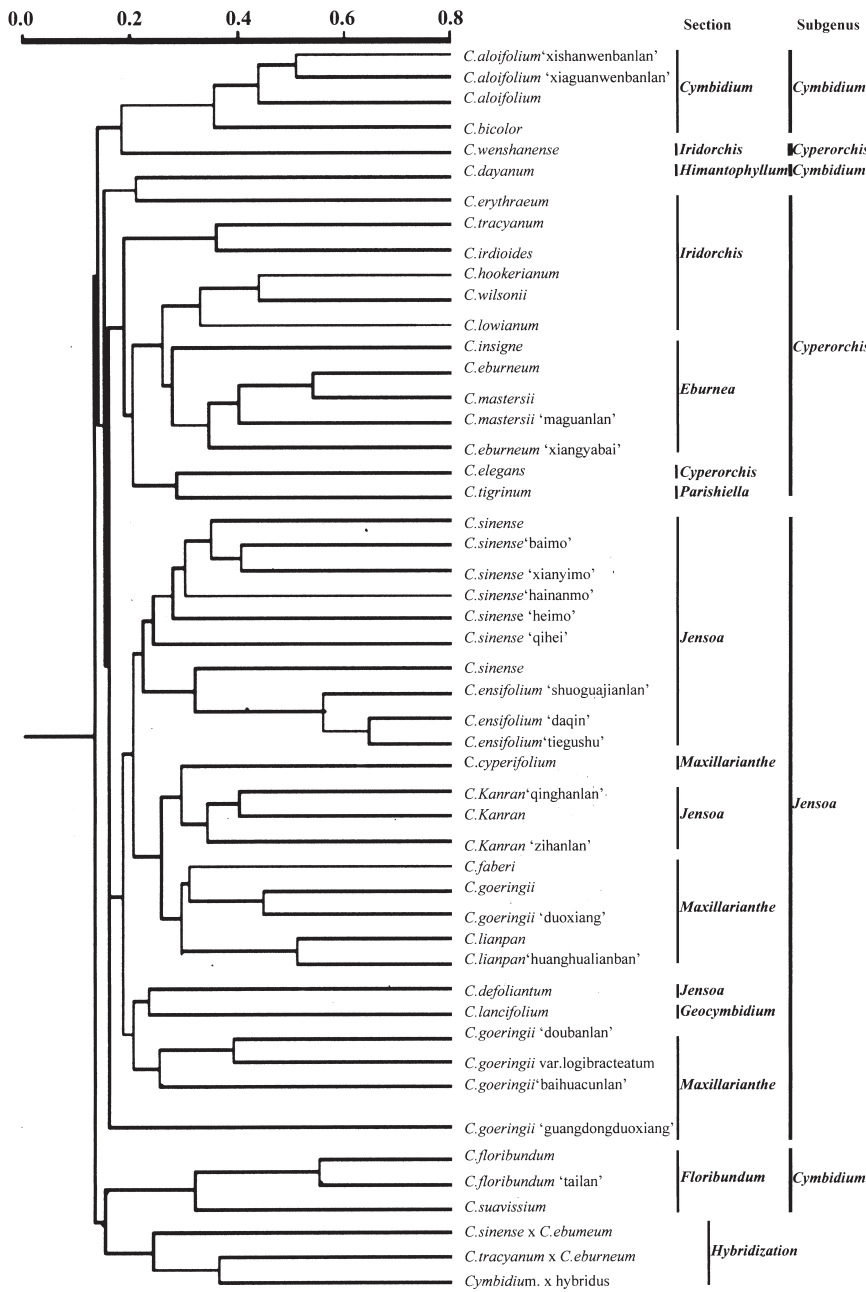


Fig. 4. UPGMA dendrogram of 50 *Cymbidium* species and cultivars in China based on Jaccard's coefficient obtained from 12 RAPD primers.

*Pachyrhizanth*. Comparison among the classifications was based on morphology [9], ITS sequence [27] and the RAPD UPGMA tree (Fig. 4). The phylogenetic trees from ITS and RAPD support the classification of subgenus *Cyperorchis* and *Jensoa*, which appeared monophyletic, only the *C. Wenshanense* is located out of subgenus *Cyperorchis* in RAPD clad tree; three sections of subgenus *Cymbidium* appeared polyphyletic, being split into several clades and intermixed with the main subgenus. However, some apparent incongruence could be noted between the ITS and RAPD results, these could be compared and viewed again to be used in phylogenetic studies.

RAPD results support traditional sectional classification of subgenus *Cymbidium* (section *Cymbidium*, *Himantophyllum* and *Floribundum*), Subgenus *Cyperorchis* (*Iridorchis* excluding *C. tracyanum* and *C. iridioides* *C. cyperorchis*, sections *Eburnea* and *Parishiella*), Subgenus *Jensoa* (*Jensoa*, Section *Maxillarianthe*, *Geocymbidium* and *Pachyrhizanth*).

## CONCLUSION

The tree generated from ITS sequences and RAPD data is only partially congruent with the current taxonomic classification of the genus *Cymbidium*, but the section classification is more congruent than the subgenus classification. Phylogenetic relationship of *Cymbidium* based on ITS [21, 27] and plastid *matK* [27] could only partly define some subgenus relationship of the genus, but it was not adequate enough to re-evaluate accurately the traditional taxonomic classification of the genus *Cymbidium* because of the low level of variation of ITS and *matK* genes. More sensitive molecular markers were tried to review the genetic diversity among cultivars and the phylogenetic relationship, which confirmed that geographic isolation could be the original source of genetic diversity in *Cymbidium* and some show phylogenetic relationship of *Cymbidium* from ITS data.

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# Genome Evolution and Population Biology in the Orchidaceae

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## ABSTRACT

Orchidaceae, the largest angiosperm family, is not at the forefront of genomic evolution research since many of the leading approaches in the study of plant genomes has not been applied to this group. The information on chromosome evolution is reviewed in this chapter, with emphasis on the importance of polyploidization in generating new genome sizes and gene pools. The recent advances in molecular phylogenetics of Orchidaceae at several hierarchical levels are gradually providing a framework for testing hypotheses on the evolution at both the morphological and genomic levels. Evolutionary phenomena from the origin of higher taxa down to the microevolutionary processes occurring at the population level have been assessed. The latter area is experiencing fast changes due to the incorporation of all the new molecular techniques developed in the recent decades. Most population biology studies with molecular markers were conducted on terrestrial orchid species, with fewer studies on epiphytic and lithophytic species.

**Key Words:** Molecular markers, chromosome, phylogeny, population structure, polyploidy, genetic diversity

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## INTRODUCTION

The main word in the title, “genome”, needs a conceptual/historical commentary before the genome evolution of the Orchidaceae Jussieu proper is addressed [sources of historical information in 54, 62, 95, 96], because genomic evolution will be discussed at several levels. The word genome had been associated with the concept of chromosome, and replaced later by the concept of germplasm, created by August Weissman in the 19th century to contrast it against the soma. The germplasm was the substance, and also the part of the body inside the gonads of the animals carrying this substance, responsible for the transmission of hereditary characters to the next generation. In 1869, Johann Friedrich Miescher, discovered a phosphorated, nitrogenated substance in leukocyte nuclei of pus, which he named *nuclein*, and in 1874 discovered that nuclein was composed of two moieties, the first being proteic, and the second viscous and acid, which he called *nucleic acid*.

The existence of the cell nucleus was discovered in an orchid prior to any other organism, by Robert Brown in 1831 [7]. The fact that chromosomes contained nucleic acids was discovered in 1923, when Robert Feulgen applied his coloration method chemically specific for the oxygen atom linked to the 2' carbon of deoxyribose (developed in 1914) to the microscopic observation of tissues.

In 1866 Mendel discovered that heredity was controlled by unitary factors, later called genes. By comparing gene segregation assessed in crossings with chromosome segregation under the microscope, Theodor Boveri and Walter Sutton independently proposed, in 1902, the Chromosome Theory of Heredity.

After the identification of the chromosomes as the carrier of genes, in the beginning of the 20th century, “genome” was introduced with the meaning of the ensemble of all the chromosomes, or of all genetic material. The word “genome” could, and still can, be applied at both the individual and the species level, and later began to be used for organelles, including the nucleus. This word is derived from the contraction of the common Greek word “*genos*” (clan) with the scientific word of Greek origin “chromosome” and, in the beginning of its history, referred essentially to the chromosome set of an individual, or of a species. See, for instance, the titles of such classical works as [143] and [71],

respectively, “Genome-analysis in *Brassica* with special reference...” and “Genomanalyse bei *Triticum* und *Aegilops*”. In the DNA era, from 1950 onwards, “genome” was finally associated with DNA.

In the context in which the term was created, “genome” was the set of chromosomes characteristic of one or several related species. The method used for assessing species relatedness consisted primarily of the observation of chromosome pairing in the meiosis of hybrids between diploid species, belonging either to the same genus or to close genera [134]. Pairing degree ranges from null to perfect. A perfect or nearly perfect pairing indicates that species which are crossed belong to the same **genomic group**, which receives an arbitrary capital letter as its symbol (A, B, C, ...) [70]. The first two species to be investigated generally are assigned to genomic group A if their chromosomes turn out to pair perfectly. Otherwise, one species is assigned to genomic group A and the other to genomic group B, and so on. In the hybrid, each chromosome from one species pairs with the corresponding chromosome in the other species. The method assumes that overall genetic differentiation along the chromosomes is reflected in the degree of their inability to pair. Although in most cases the separation into genomic groups is discrete, sometimes intermediate degrees of pairing are found, and in such cases the capital letter is superscripted with a low-case letter in the symbol ( $A^g$ ,  $A^l$ , etc...) [94]. Only a small proportion of the plant genera, however, has been studied at a level of detail sufficient for generating data about hybrid meiosis.

The genome is **compartmentalized** within the cell. If wheat or rice breeders are asked about how many genomes their target taxon is constituted of, they will probably take the question in the genomic group context, which relates only to nuclear DNA, and will answer maybe “two, B and C”, or “three, A, B and D”, but researchers of other fields, particularly molecular biology of photosynthesis, cell respiration or signal transduction, might think of three genomes for nearly all plants: the nuclear genome, the chloroplast genome (also called *plastome*, or cp DNA) and the mitochondrion genome (the *chondriome*, or mt DNA). It may be practical to refer to different genomes within the same cell in protist species with more than one nucleus, or in bacteria, which often have a small, facultative circular DNA molecule (the plasmid, accompanying the main circular DNA molecule).

The third main nuance of the word “genome” appeared with the application of the slab gel electrophoresis technique for DNA sequencing, which was proposed nearly simultaneously in two papers, [85] and [110]. Automation was the technical element necessary for the jump from the relatively small projects involving a few thousand bases, such as the rice chloroplast genome sequencing [65], to genome-wide, high-throughput projects, which came to be known as “**genome projects**”. Although the Human Genome Project has been the most widely publicized, the genomes of many animal ([www.ri.bbsrc.ac.uk/cgi-bin/arkdb](http://www.ri.bbsrc.ac.uk/cgi-bin/arkdb), <http://flybase.bio.indiana.edu>), plant ([http://www.nal.usda.gov/pgdic/Map\\_proj](http://www.nal.usda.gov/pgdic/Map_proj)) and microorganism (<http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl>) species have been completed or are under way. The abundant information generated by genome projects enabled the rise of the subdiscipline of genomics, which has been gradually incorporating a number of objectives. Presently, the main objectives of genomics are to assemble physical (nucleotide) and genetic (recombination) maps of the genome, with emphasis on expressed genes, often identified partially as ESTs; to annotate the genes found, i.e. compile the information available on their function, expression, natural polymorphism and homology with other genes, both in the same or in other species; to study synteny, or the conservation of gene order across species [19, 49]. A gene is expressed through RNA and protein, and its function is described by its insertion in the metabolic pathways of the organism. Thus, the genome-wide study of gene expression and function entailed the creation of the ancillary disciplines of transcriptomics and proteomics, which analyze the distribution of the RNAs (the transcriptome) and proteins, (the proteome) respectively, produced in the different tissues. The integrated study of the gene functions in a genome-wide context is called metabolomics. Gene structure, expression and functions have been the objective and praxis of geneticists since the beginning of the 20th century, but until the 1990s particular projects were limited to a small number of genes, traits and pathways. The appearance of genomics was the result of a drastic change in the scale of data accumulation capabilities. We have given ourselves the objective of surveying genome evolution in a broad sense. The material ranges from macroevolutionary approaches, such as family-, tribe-, subtribe- and genus-level phylogenetic studies, through chromosome evolution to microevolutionary works addressing basic processes and population

structure based on molecular markers. Given the incipience of genomic studies in orchids, macroevolution in this group is still heavily inclined towards straight systematics.

## CHROMOSOME EVOLUTION

### General Aspects

Chromosome counts in Orchidaceae range from  $2n=12$  in *Psigmorchis pusilla* [44] to  $2n=168$  in *Oncidium* Sw. [120, 121]. The commonest numbers are  $2n=38$ , 40 and 42, but all the even numbers from 24 to 60 are well represented [136]. There are very few subtribes of Orchidaceae with constant chromosome numbers (Table 1). Usually, numerical variation is found not only within tribes and subtribes, but also within genera and species. Even the tissues or cells of a single individual may show variation, which is called polysomaty (examples in *Cymbidium* Sw. [47]; *Dendrobium* Sw. [67]; *Phalaenopsis* Blume [86]). The mechanistic cause of polysomaty is endoreduplication, the DNA synthesis without cell division, and its occurrence and rate range across tissues and organs. In *Cymbidium* it is absent from ovaries, rare in leaves and common in roots and flowers. In *Dendrobium* and *Phalaenopsis* it is also common in leaves. Endoreduplication begins after a few weeks in *Papilionanthe hookeriana* (Rchb.f) Schltr. (syn. *Vanda hookeriana*) X *Papilionanthe teres* (Roxb.) Schltr. (syn. *Vanda teres*) embryos [80] and continues throughout life producing 2C to 16C nuclei in adults, distributed unevenly in the tissue types. Root tips are generally endopolyploid in this material and so pose a problem for germ line chromosome counting. Apparently, polysomaty does not have a direct relation to heritable numerical variation because endoreduplication usually occurs in cell lines without a potential for shoot regeneration or gamete formation. The genetic potential for endoreduplication, with the consequent increase in vigor, is probably of adaptive value and must be subjected to selection.

The evolution of the chromosome numbers in a given clade may occur by aneuploidy, euploidy or, more commonly, both. In orchids, the relative weight of each cause in the evolution within the subfamilies is not clear enough for consensus. Five, 6, 7, 10 and 11 have been suggested as the base haploid numbers in various occasions [42]. Part of the problem arises from the possibility that the number of chromosomes may increase through real duplication of the chromosome set (euploidy),

**Table 1.** Phylogenetic distribution of chromosome numbers (2n) and genome sizes (in pg/2C) over suprageneric taxa of Orchidaceae (*see notes at the end of the table*)

Subfamily <sup>a</sup>	Tribe	Subtribe	2n <sup>b</sup>	Mean genome size (pg/2C)
Apostasioideae Cyripedioids [5]			48, 96, 144	
			18, <u>20</u> , 21, 22, 24,	12.2-69.1 [37]
			26, 27, 28, 29, 30,	
			32, 34, 36, 37, 40,	
			42, 48, 52, 54, 56	
			[37]	
Vanilloids		Pogoniinae	18, 19, 20, 21, 23, 24	
		Galeolinae	28, 30	
		Vanillinae	28, 30, 32	15.19; 14.45
Orchidooids [74]	Orchideae	Orchidinae	20, 24, 30, 32, 36,	
		[12, 13, 38]	38, 40, <u>42</u> , <u>44</u> , 46,	
			48, 63, 64, 72, 73,	
			80, 84, 85, 100, 120,	
			126 [15]	
		Habenariinae [13]	14, 16, 28, 30, 32,	
			34, 36, 38, 40, <u>42</u> ,	
			44, 46, 48, 62, 64,	
			76, 84, 126	
			36, 38	
Diseae	Diseae	Disinae		
		Satyrinae	36, 41, 42, 82, 164	

Table 1 contd.

Diurideae (1) [75, 76]	Chloracinae	16
Cranichideae [109] Goodyearinae	Pterostylidinae	42, 50
	Pachyplectroninae	?
		20, 22, 24, 26, 28, 30, 32, 40, 42, 44, 48, 50, 56, 100, 144
Diurideae (2) [76]	Prescottiiinae	?
	Spiranthinae	24, 26, 28, 30, 32, 35, 36, 44, 46, 56, 60
	Cranichidinae	46
	Acianthinae	38, 40, 44, 54
	Prasophyllinae	44
	Caladeniinae (1)	(for the taxon as a whole) 38, 42, 44, 46, 48
	Cryptostylidinae	42, 56
	Diuridinae	38, 56
	Caladeniinae (2)	(for the taxon as a whole) 38, 42, 44, 46, 48



“lower” Epidendroids	Anomalous Nervilieae	Drakaeinae	40, 44		
		Thelymitrinae	24, 26, 32, 56		
	Diceratosteleeae		-	20, 36, 40, 54, 72,	
				108, 144	
			?		
		Triphoreae	44		
	Palmorchideae			40, 56, 58, 60	
			?		
		Limodorinae	20, 24, 32, 34, 36,		
			38, <u>40</u> , 42, 44, 46,		
“higher” Epidendroids	Epidendreae (1)		48, 56, 60		
		Listerinae	20, 32, 34, 35, 36,		
			38, 40, 42, 46, 56		
			54		
	Coelogyneae	Sobralinae			
		Coelogyneinae [52]	38, 40, 42, 44, 76,		5.48
	Arethuseae (1)		80, 120		
			Thuniinae	38, 40, 42, 44	
			Bletinae (1)	(for the taxon as a	
				whole): 20, 26, 28,	
			30, 32, 36, 38, 40,		
			<u>42</u> , 44, 46, 48, 50,		
			52, 54, 58, 60, 72		

Table 1 contd.

Epidendreae (2)	Glomerinae (1)	(for the taxon as a whole) 38, 40, 46	11.38
Arethuseae (2)	Arethusinae	40, 44	
	Bletiinae (2)	(for the taxon as a whole): 20, 26, 28, 30, 32, 36, 38, <u>40</u> , <u>42</u> , 44, 46, 48, 50, 52, 54, 58, 60, 72	
Epidendreae (3)	Coelinae	40	
	Laelinae (1)	42 [for <i>Dilomilis</i> only]	
	Pleurothalidinae [103]	20, 30, 32, 34, 36, 38, 40, 42, 44, 64	
Arethuseae (3)	Bletiinae (3)	(for the taxon as a whole): 20, 26, 28, 30, 32, 36, 38, <u>40</u> , <u>42</u> , 44, 46, 48, 50, 52, 54, 58, 60, 72	
		30, 32	
		(for the taxon as a whole): 18, 20, 30, 32-35, 36, 38, 40, 41, 57	
Podochileae	Thelasiniae	30, 32	1.53-4.23 (for <i>Dendrobium</i> alone)
	Dendrobiinae (1)	(for the taxon as a whole): 18, 20, 30, 32-35, 36, 38, 40, 41, 57	
	Podochilinae	38, 40	

Table 1 contd.

Malaxideae	Eriinae	18, 20, 24, 34, 36, 38, 40, 42, 44, 46, 66	
		20, 26, 28, 30, 32, 34, 36, 38, 42, 44, 46, 72, 80	
		(for the taxon as a whole): 18, 20, 30, 32-35, 36, 38, 40, 41, 57	1.91;
		36, 38, 39, 40, 42, 57, 60, 80	5.35;
Dendrobieae	Dendrobiinae (2)	(for the taxon as a whole): 18, 20, 30, 32-35, 36, 38, 40, 41, 57	
		36, 38, 39, 40, 42, 57, 60, 80	
		(for the taxon as a whole): 18, 20, 30, 32-35, 36, 38, 40, 41, 57	
		(for the taxon as a whole) 38, 40, 46 ?	
Epidendreae (4)	Glomerinae (2)	24, 28, 32, 36, 40, 42, 46, 48, 50, 52, 54	
Cymbidieae (1)	[Govenia]		
Calypsoeae			

Table 1 contd.

Arethuseae (4)	Chrysiinae Bletiinae (4)	?  (for all the clades of the taxon): 20, <u>26</u> , 28, 30, 32, 36, 38, <u>40</u> , <u>42</u> , 44, <u>46</u> , <u>48</u> , 50, 52, 54, 58, 60, 72	
Epidendreae (5)	Meiracyllinae Polystachyinae Laeliinae [144, 145]	?  38, 39, <u>40</u> , 80, 81  (for the taxon as a whole) 24, 38, 39, <u>40</u> , 41, 42, 44, 48, 54, <u>56</u> , 57, 60, 63, 70, 80, 90, 160	3.29; 2.12; 4.98; 4.99; 3.29; 5.97; 5.31; 8.55; 8.13; 9.29; 7.30; 2.87; 2.45; 3.51; 3.89
Vandeae	Arpophyllinae Aerangidinae  Angraecinae [28]	?  42, 44, 46, 48, <u>50</u> , 52, 54  34, 36, 38, 40, 42, 44, 46, 48, 50, 63, 76, 95	
	Aeridinae	16, 18, 20, 24, 28, 30, 32, 34, 36, <u>38</u> , 39, <u>40</u> , <u>56</u> , 69, 74, 76, 112, 113, 114, 115, 116	6.40; 9.25; 4.73; 5.53; 8.65; 6.02; 9.65; 4.19; 4.10

Table 1 contd.

Cymbidieae (2)	Cyrtopodiinae (1)	32, 38, 40, 42	16.5; 3.70; 6.31; 3.44;
	Eulophiinae	<u>32</u> , 34, 36, 38, 40, 41, 42, 44, 46, 48, 50, 52, <u>54</u> , 56, 58, 60, 66, 68, 70, 72, 74, 76, 80, 82, 94, 96, 100, 112 (for all the clades of the taxon) 32, 38, 40, 42	
	Cyrtopodiinae (2)	54, 56, 64, 68, 108, 162 (for all the clades of the taxon) 32, 38, 40, 42	
	Catasetinae		
	Cyrtopodiinae (3)	10, 14, 24, 26, 28, 30, 33, 34, 36, 37, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 59, 60, 63, 72, 84, 6, 112, 126, 1338	3.74; 4.78; 4.74; 3.85; 4.67;
Maxillarieae [150]	Oncidiinae		

Table 1 contd.

Table 1 contd.

Telipogoninae	?	
Zygopetalinae (1)	(for all the clades of the taxon)	46, 47, 48, 50, 52, 96
Cryptanthinae	?	
Anomalous	-	
Zygopetalinae (2)	(for all the clades of the taxon)	46, 47, 48, 50, 52, 96
Stanhopeinae		38, 40, 41, 42, 80
Lycastinae		38, 40, 44, 48, 50
Anomalous	-	
Maxillariinae		40, 42

<sup>a</sup>Subfamily, tribe and subtribe names are those given in Dressler [42]. The distribution of subtribes within tribes and of tribes within subfamilies follows the phylogeny hypothesis of Cameron et al. [25]: the first tribe in a subfamily is given in the same line and the other tribes follow downwards until the next subfamily is reached; an analogous arrangement is valid for subtribes within tribes. Some tribes and subtribes are polyphyletic and appear more than once. In this case they are given roman numerals in parentheses, the lowest representing the basalmost clade and the highest representing the most apical in the phylogeny in Cameron et al. [25].

<sup>b</sup>Somatic chromosome numbers (2n) in common roman type refer to Tanaka & Kamemoto [136] and references therein, which are not cited here; numbers in **boldface** refer to Dressler [42]; numbers in *italics* refer to works indicated in between square brackets. Documented natural euploids and aneuploids are included. Modal (most frequent) numbers are underlined, when applicable. Interspecific hybrids, colchiploids and commercial varieties were not included. Genome sizes in **boldface** are from Jones et al. [67].

or by centric fission (aneuploidy). In the latter case, there is some increase in the DNA content, but not proportional to the increase in number. In both cases, in the long run, approximately the same numbers could be achieved. Centric fusions, on the other hand, could reduce the  $2n$  number, causing, for instance, 20 telocentric chromosomes to reduce to 10. In spite of the great numerical diversity, a large bulk of evidence points to  $n=10$  as the base number for Cyripedioids and  $n=20$  for Epidendroids so that the latter might be tetraploids derived from a common ancestor [42]. The Apostasioids, the basal clade of the family, with  $2n$  numbers equal to exact multiples of 48, is the group least prone to aneuploid variation. It is possibly a tetraploid itself [42], in which case its ancestor ( $x=n=12$ ) might have an aneuploid relation with the ancestor of the Cyripedioids. Curiously, the chromosomes in Apostasioideae are smaller than in Cyripedioideae, which indicates evolutionary change in DNA contents.

The highest within-genus chromosome number diversity is found in *Angraecum* Bory, *Bulbophyllum* Thouars, *Calanthe* R. Br., *Cattleya* Lindl., *Dactylorhiza* (Klinge) Verm., *Epidendrum* L., *Eria* Lindl., *Eulophia* R.Br ex Lindl., *Goodyera* R.Br. in W.T.Aiton, *Gymnadenia* R.Br. in W.T.Aiton, *Habenaria* Willd., *Laelia* Lindl., *Liparis* Rich., *Listera* R.Br. in W.T.Aiton, *Microstylis* (Nutt.) Eaton, *Oncidium*, *Paphiopedilum* Pfizer, *Phalaenopsis*, *Platanthera* Rich., *Spiranthes* Rich., *Vanda* Jones ex R.Br. and *Zeuxine* Lindl. (Table 1). Variation is the rule in large genera and in many smaller genera apparent constancy may be due to incomplete sampling. Besides the loss and gain of ordinary chromosomes by aneuploidy, which is evident from the long list of almost continuous  $2n$  numbers, species vary among themselves in number of supranumerary, or B chromosomes. These chromosomes arise probably by chromosome number reduction and heterochromatinization in formerly normal chromosomes [15], and are not usually essential. They compose the karyotypes of *Coelogyne* Lindl. [136], *Dactylorhiza* Neck. Ex Nevski [15], *Dendrobium*, *Eria*, *Goodyera*, *Listera*, *Microstylis*, *Ophrys* L., *Paphiopedilum*, *Pleione* D.Don, *Spiranthes* and *Tainia* Blume. Curiously, many of these genera are also among the most diverse in terms of  $2n$ , perhaps because an increase in the total number of chromosomes facilitates the transformation of a higher number in supranumerary.

Polyploidy often has the effect of enhancing size, color, thickness and durability. As such effects are desirable to some extent in the ornamental

market, polyploidy is normally sought by producers, who may just select chance mutants or induce them with colchicine, in which case they are called colchiploids. Much of the cytological diversity in the most popular genera, such as *Cattleya*, *Cymbidium*, *Dendrobium*, *Paphiopedilum*, *Phalenopsis* and *Vanda* is not found in natural populations [136].

## Genome Size Evolution

Chromosome number may vary without a proportional change in genome size. Conversely, it may remain constant across species that show considerable genome size differences. In *Dendrobium*, for instance, genome size varies three-fold among species while the  $2n$  number of chromosomes remains almost constant at 38 (Table 1) [67]. On the other hand, *Neofinetia falcata* (Thunb.) Hu and *Oncidium sphacelatum* Lindl. have nearly the same amount of DNA per  $2C$  (4.73 and 4.74 pg, respectively) but very different  $2n$  numbers (38 and 56, respectively) [67]. The nature and evolutionary meaning of the variable DNA is unknown. Ideally, both variables should be studied together, but unfortunately the genome size of very few species is known (Table 1).

## Evolution by Allopolyploidy

Polyploidy involves the increase in the numbers of whole haploid chromosome sets, which may or may not be of the same species. In the former case, called autopolyploidy, more than two homologues may pair in the meiotic prophase, and this may cause irregularities in spore formation and sterility, but after selection, meiosis may stabilize. *Serapias lingua* L., for instance, is considered an autotetraploid derived from *S. parviflora* Parl. ( $2n=36$ ) [15]. In the latter case, called allopolyploidy, the component species generally belong to different genomic groups. Tetraploids, which have two haploid sets of one type and two of another (such as AABB), are called amphiploids. If the chromosomes of the parent species are too similar, they can also form multivalents in prophase I. There are basically two mechanisms leading to allopolyploidy. Unreduced gametes from different species can form a polyploid zygote, which grows into an allopolyploid adult; or an interspecific hybrid may be formed from two normal gametes from different species and become polyploid during its vegetative growth. Allopolyploidy is probably a common speciation mechanism among orchids [128, 130]. *Dactylorhiza insularis* (Sommier ex Martelli) Landwehr, for instance, was shown to be



a hybrid of *D. romana* (Sebast. & Mauri) Soó and *D. sambucina* (L.) Soó, which can also form autotriploids by the union of  $n$  and  $2n$  gametes [15]. Many species of the Cypripedioids, Orchidoids and Epidendroids with  $2n$  equal to or around 40, 60 or 80 [136] are probably allopolyploids.

In determining the putative parent species several methods should be used. The first method is chromosomal morphological analysis, including a comparison of the karyotype among the three species (hybrid and putative parents). In the orchid genus *Spiranthes*, several allopolyploids with  $2n=74$  have been found to be the result of hybridization between diploid species with  $n=15$  and  $n=22$  ( $2 \times [15+22]=74$ ) [130].  $2n$  numbers, fundamental numbers and distribution of chromosome types, as well as banding patterns and fluorescence techniques can be used to identify similarities.

A second method is the comparison of the population genetic structure of parents and hybrids. When the parent species are homozygous for different alleles at the same locus, the allopolyploid will display genotype additivity [10] with “fixed heterozygosity” which appears as two isozyme bands on a gel [130 for *Spiranthes*]. By contrast, the different alleles of the parents will not be fixed in autopolyploids, but will segregate polysomically, producing genotypes of the types AAAA, AAAa, AAaa, Aaaa and aaaa (‘A’ and ‘a’ are alleles). If an allotetraploid shows a number of different alleles per locus higher than four, an additional conclusion can be drawn that more than one hybridization event originated the taxon, because two alleles per locus is the maximum each parent can contribute in one single event. That is the case for *Spiranthes diluvialis* Sheviak [10]. Similar conclusions were reached in relation to the origin of the allopolyploid orchid *Platanthera huronensis* Lindl., whose probable progenitors are the diploids *Piperia dilatata* (Pursh) Szlach. & Rutk. (syn. *Platanthera dilatata*) and *Platanthera aquilonis* Sheviak [147]. The allopolyploid shows five different chloroplast RFLP (restriction fragment length polymorphism) haplotypes corresponding to different hybridization events between the diploid parents. The long-distance dispersal by the minute seeds accounts for the wide geographic distribution of the haplotypes in North America.

A third possible method is the comparison of nucleotide sequences of homologous genes retrieved by PCR from the allopolyploid hybrid and the parents. If the region sequenced is moderately conserved, the diploids are likely to have only one haplotype, which may be different in each species.

An allotetraploid would have then two different haplotypes (considering a nuclear gene), which should be first separated by cloning in bacteria. Phylogenetic analysis is able to indicate which parent is more likely to have contributed each allopolyploid haplotype (e.g. Ge et al. [48], for rice). If cloning does not separate the allopolyploid haplotypes, the electrophoregram shows sequence heterogeneity and makes the analysis more difficult and more dependent on chromosome and population genetic analysis [135]. A complementary strategy is reconstructing a cpDNA- or mtDNA-based phylogeny, so that the species closest related to the allopolyploid can be identified as the maternal parent [97]. The parents of the allopolyploid American orchid *Spiranthes diluvialis* were identified as the diploids *S. romanzoffiana* Cham. (the mother) and *S. magnicamporum* Sheviak (the father) by a combination of methods [135].

Sometimes the complexity of the data defies an easy explanation and even a combination of methods is unable to shed light on the origin of an allopolyploid. ITS (internal transcribed spacer) sequencing, AFLPs (amplified fragment length polymorphism) and plastid restriction fragments were analyzed by maximum parsimony methods, in addition to UPGMA (unweighted pair-group methods with arithmetical averages) analysis of the AFLPs, in an attempt to identify the parents of the supposedly allohexaploid orchid *Calopogon oklahomensis* D.H. Goldman, but the results precluded a conclusion about its origin [50]. It is not absolutely clear whether the species is really a hybrid or not. If it is, it may be an ancient, much modified allopolyploid, which has already accumulated specific mutations.

Genomic groups and their notation have been developed and used in Orchidaceae mostly in the genome breeding literature, to describe the genomic formulae of commercial, artificial hybrids. Curiously, none of the natural allopolyploids in *Spiranthes* and *Calopogon* R.Br. in Aiton cited above has been described in terms of genomic formulae. Genomic groups have been best studied perhaps in *Dendrobium*, where approximately each section of the genus receives a different capital letter. Thus, section Spathulata is symbolized by C (it was formerly called Ceratobium), Eugenanthe by E, Latourea by L, Formosae by N and Phalaenanthe by P [7]. Species within sections are designated by the section's capital letter superscripted with a species-specific lowercase letter. The genome of *D. gouldii* Rchb.f., for example, is C<sup>g</sup>, while that of *D. bigibbum* Lindl. (syn. used in ref. *D. phalenopsis*) is P<sup>p</sup>, so that the fertile allopolyploid hybrid variety

obtained from them is symbolized by  $P^P P^P C^g C^g$  and has disomic segregation.

## MOLECULAR EVOLUTION AND PHYLOGENY

The Orchidaceae Jussieu are the largest angiosperm family, with an estimated number of species ranging from 18,000 to 35,000 [25, 107, 130] and nearly 850 genera, divided into five main clades [25]: Apostasioids, Cypripedioids, Vanilloids, Orchidoids and Epidendroids. Although they do not correspond exactly to the formal subfamilies named by Dressler [42], the differences are not striking and these informal clade names are likely to be referred soon by their Latinized forms. The subfamilies are subdivided into tribes and subtribes (Table 1), and the general structure of the family fits the “hollow curve” [115] displayed by many other families, indicating a strong asymmetry in the distribution of species within genera, genera within subtribes, and subtribes within tribes. In phylogenetic terms, this means that many short branches are concentrated in some apical clades and many long branches in the basal clades.

### Descriptive Knowledge of Orchid Genome at the Nucleotide Sequence Level

In spite of its size, the Orchidaceae has not been the focus of the genomic revolution mainly because of its relatively minor economic importance when compared to food crops. A simple comparison between orchids and grasses, shows that molecular research on the former lags way behind. A search for DNA sequences in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) retrieved 6,755 records of Orchidaceae, while 6,202,444 records represented Poaceae. There is no orchid genome sequence project or any genetic mapping project, or even a wide-scope EST (expressed sequence tag) sequencing project registered, either completed or under way. As a consequence of this lack of basic information about genes in orchids, there is a marked tendency in evolutionary studies to use regions located between universal primer sites [98]. In Poaceae, by contrast, beside the ITS and chloroplast generic regions, there has been an increasing use of more specific primers, such as those amplifying alcohol dehydrogenase [48] or starch synthase genes [84], which have the advantage of being single-copy genes or being present in few copies in the genome.

Among the main genes already described at the molecular level in orchids are those directly involved with floral biology [156]. Using mainly *Dendrobium*, *Phalaenopsis* and *x Aranda* Auct. systems, several genes were identified, such as (1) those belonging to the *APETALA1/AGL9* subfamily and the *DOMADS* series of the MADS-box; (2) those affecting flower pigmentation, such as dihydroflavonol 4-reductase (DFR), chalcone synthetase, flavanone 3-hydroxylase and Phe ammonia-lyase, whose regulation, though, is not well understood; more recently, the cDNA (complementary DNA) of a flavonoid-3', 5'-hydroxylase gene, which generally produces blue and purple flowers, was isolated from *Phalaenopsis* [129]; (3) O39, which may be involved in the pollination-dependent initiation of ovule development, a process unique to orchids; and (4) the genes involved in ethylene production, and consequently, in ovary maturation, such as ACC (1-aminocyclopropane-1-carboxylic acid) synthase and ACC oxidase. The importance of the molecular dissection and evolutionary studies of these genes can be measured by the centrality of the flower biology in the divergence of Orchidaceae.

The *ndhF* chloroplast gene, which encodes a component of NADH (Nicotinamide Adenine Dinucleotide) dehydrogenase, has been isolated from the orchid *Restrepia* Kunth in F.W.H.von Humboldt, A.J.A.Bonpland & C.S.Kunth (Epidendreae, Pleurothaliaceae) and sequenced [88] but no comparison has been done with other orchids, so the utility of the gene for phylogenetic studies within this family remains unclear. Another gene involved in the energy metabolism, *sps1* (sucrose-phosphate synthase), was isolated from the orchid cultivar *Oncidium* Goldiana (Epidendreae) by RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) [78]. The gene is 3,183 bp long, its correspondent polypeptide has 1,061 amino acid residues and a peculiar expression pattern, producing more RNA (ribonucleic acid) in the flowers, than in the leaves, in contrast with *sps* genes found in other families. A second gene affecting the sucrose metabolism, *Msus1*, was isolated from the multigenus orchid hybrid *Mokara* Auct., and is classified as a class I sucrose synthase [79]. This is one of the very few true studies in molecular evolution concerning an orchid. The cDNA, with an open reading frame of 2,447 bp, coding a 816-residue polypeptide, was aligned with similar genes isolated from other species and subjected to a phylogenetic analysis, which produced four clear clades artificially grouped into three classes: (1) the monophyletic Class I, including only monocots, (2) its sister-group Class II, composed of dicots, and (3) the

paraphyletic Class II, composed of dicots. The tree contains conspecific paralogues distributed sometimes through two clades, and often has two or more conspecific paralogues in the same clade, indicating that the multigene family evolved by recent (within the species history) and remote (before the species diverged) gene duplication. The conspecific paralogues correspond to isozymes found in electrophoretic studies and sometimes are expressed in different tissues of the same organism, providing metabolic flexibility to the species. The *Mokara sus1* gene is, within the limited monocot sampling available for analysis, closest related to the *Tulipa sus1* and *sus2* genes and is expressed in growing leaves. A more detailed study of gene expression in orchids was performed in *Dendrobium*. *DSCKX1*, a gene coding for a cytokinin oxidase, was sequenced after isolation from *Dendrobium Sonia* [154] and its promoter analyzed by fusing a reporter gene to promoter fragments [155]. The promoter contains regions for the spatial and temporal control of the cytokinin oxidase production and for cytokinin binding, which is necessary for gene transcription.

## Phylogenetic Structure of Orchidaceae

A molecular phylogeny generates a hypothesis about the relationships between taxa, which can be used as a frame for devising further research about character evolution, speciation, chromosome and genome size evolution, syntenic, and gene family evolution. Molecular phylogenetic studies in the Orchidaceae have been done at several levels, from family [25, 32] through tribe and subtribe to genus (Table 1). The number of tribes, subtribes and especially genera, which have been the focus of these studies, however, is very limited. Mark Chase of the Royal Botanic Gardens and his collaborators have done most of the family- and tribe-level works. The most comprehensive phylogeny of the family was based on sequences of the plastid gene *rbcL* (RuBisCo large subunit gene) [25], a gene with a remarkably conserved length, thus permitting a straightforward alignment. This study included 171 species and most of the subtribes were represented by 1-6 species. According to the *rbcL* phylogeny, the family is organized in five subfamily-level clades (i.e. monophyletic groups), viz. Apostasioids, Cypripedioids, Vanilloids, Orchidoideae and Epidendroideae, from the base to the apex. The phylogeny is congruent in many aspects to the most accepted systematic structure of the family [42], but reveals that some taxa are not monophyletic as circumscribed by Dressler. Among the paraphyletic taxa are the genus

*Cleistes* Rich. ex Lindl., tribes Cranichideae, Diseae, Orchideae, Calypsoeae, and Cymbidieae, and subtribes Habenariinae and Limodorinae. The following taxa are polyphyletic: tribes Diurideae, Arethusae and Epidendreae, and subtribes Bletiinae, Glomerinae, Dendrobinae, Laeliinae, Stanhopeinae, Zygopetalinae and Cyrtopodiinae. While these defects mean further restructuring work for systematists, they pose interesting questions to the evolutionist regarding convergence and heterogeneous rates of change.

The combined phylogenetic analysis of plastid regions *matK* plus *trnL-F* in the subfamily Orchidoideae [76] confirmed the polyphyletic nature of tribe Diurideae. If, however, Chloraeinae and Pterostylidinae are removed, Diurideae is rendered monophyletic and is shown to be composed of three major clades: the first one comprises mostly Drakaeinae, Thelymitrinae, Cryptostylidinae and Diuridinae, the second has only the core Caladeniinae, and the third has Acianthinae and Prasophyllinae. All these subtribes are monophyletic and most have good bootstrap support. The topology based on *matK* and *trnL-F* shows the subfamily Orchidoideae organized in three clades. The basalmost is a combination of Orchideae and Codonorchideae. The second is subdivided in the Diurideae as defined above and a clade, informally called “spiranthid”, composed of the Cranichideae and its sequential sisters Pterostylidinae, Megastylidinae (pro parte), and the core Chloraeinae. The genus *Megastylis* (Schltr.) Schltr. is grossly polyphyletic, part of it really belonging to the spiranthids while *M. rara* (Schltr.) Schltr. is placed within Diurideae. The selective forces which caused the morphological convergence lying at the root of this systematic confusion are unknown.

In order to refine the phylogeny of the “spiranthids” and define the limits of Cranichideae, a joint analysis using the plastid regions *rbcl*, *matK* and *trnL-F* plus the nuclear ribosomal ITS, was performed [109]. Pterostylidinae, Megastylidinae, Chloraeinae, Diurideae, Codonorchideae and Orchideae, the outgroups of the analysis, are the sequential sisters to spiranthids, in increasing order of relatedness. The Cranichideae sensu Dressler [42] is actually composed of ten major clades, the five apicalmost of which are redefined as the Spiranthinae. Apart from Spiranthinae, the core spiranthids is composed of Pachyplectroninae, Goodyerinae, Galleottielinae, Manniellinae, Prescottiinae and Cranichidinae. The indels affecting nucleotides normally necessary for proper functioning in the *matK* gene, suggest that this region may have become a pseudogene.

In Cameron et al. [25], the Maxillarieae, one of the largest tribes of the family, was problematic, with Stanhopeinae and Zygopetalinae being polyphyletic. The use of ITS1 and 2, *matK*, the *trnL* intron and *trnL-F* in a broader sample of the tribe considerably clarified the relationships between genera and permitted a better assessment of the monophyly of the subtribes. Six monophyletic subtribes appear on the phylogeny: (1) Eriopsidinae, sister to the rest; (2) Oncidiinae, sister to a clade formed by (3) Stanhopeinae, (4) Coeliopsidinae, (5) Maxillariinae, and (6) Zygopetalinae. *Zygopetalum* Hook., which had been placed in a clade separate from *Dichaea* Lindl., *Koellensteinia* Rchb.f. and *Cryptarrhena* R.Br. in the *rbcL* phylogeny, is joined in a monophyletic Zygopetalinae. Cryptarrheninae is incorporated in Zygopetalinae. All the genera of Stanhopeinae represented by at least two species in the study were monophyletic, and *Lycomormium* Rchb.f., which had been responsible for the polyphyly of the subtribe, is now placed as sister genus to *Coeliopsis* Rchb.f. in Coeliopsidinae.

One of the most spectacular cases of polyphyly in orchids was revealed in *Pleurothallis* R.Br. in W.T.Aiton, in a molecular phylogenetic reconstruction of subtribe Pleurothallidinae [103]. This phylogeny used the ribosomal ITS region and 5.8S gene for the entire taxon sample, complemented by the plastid genes *matK*, *trnL-F* and the *trnL* intron for subsets of genera and produced a structure composed of nine major clades. *Pleurothallis*, as commonly circumscribed, appears in five of these clades, showing extensive polyphyly. Paraphyly is a possible problem in genera *Dracula* Luer, *Myoxanthus* Poepp. & Endl. and *Restrepia* [103], unless nomenclatural changes are proposed. Genus *Dilomilis* Raf., usually included in Laeliinae [42] was found to be a source of problems in the *rbcL* Orchidaceae phylogeny [25] because it appeared as a sister-group of Pleurothallidinae, in a position in the tree that made Laeliinae polyphyletic. *Dilomilis*, together with *Neocogniauxia* Schltr. in I.Urbán, appears close enough to Pleurothallidinae to be considered as the basal part of it [103]. This transference is also supported by molecular studies performed in Laeliinae [144, 145], which suggest that *Dilomilis* should be removed from this subtribe.

This sequence of works illustrates how the fine phylogenetic structure of Orchidaceae has been improving from top to bottom with hierarchical increases in taxon sampling towards the different subfamilies, tribes, subtribes and genera, actually encompassing most of the relevant studies at levels higher than genus. In all cases, the phylogeny can be



used as an independent historical indicator upon which one can map morphological and ecological traits in order to find evidence for evolutionary phenomena, such as convergences, atrophies, adaptive radiations and other speciation patterns [14 and 68 for *Disa*; 52 for *Coelogyne*; 38 for *Orchys* Tourn. Ex L.; 140 for *Oncidiinae*; 73 for *Bifrenaria* Lindl. and related genera; 157 for *Kitigorchys* Maek.; 142 for *Phalaenopsis*; 28 for *Angraecinae*].

## MOLECULAR MARKERS AND POPULATION BIOLOGY IN ORCHIDS

To understand the microevolutionary processes such as genetic drift, natural selection, hybridization, mutations, associated with geographical distribution and human activity, molecular markers, such as isozymes, RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), SSR (simple sequence repeats), ISSR (intersimple sequence repeats) and AFLP (amplified fragment length polymorphism), are all important tools utilized in many studies of plant population biology.

The Orchidaceae is considered an excellent group for studying evolution [42]. The sheer size of the family and its numerous taxonomic ambiguities suggest that genetic resources are substantial, evolutionary potential is high, and this diversity is far from static [2]. Population biology studies have been conducted with orchid species using several molecular markers, such as isozymes, RAPD, SSR, AFLP, chloroplast DNA sequencing, chloroplast minisatellite, ISSR, with different objectives: a) the study of levels of genetic variation within and among populations and species, and patterns of population structure [3, 17, 22, 23, 29, 30, 55, 57, 66, 83, 87, 112, 113, 118, 125, 151]; b) effect of genetic drift or small sized populations on genetic variation [20, 30, 39, 66, 118, 125, 131, 139, 146]; c) phylogenetic relationships among species [16, 35, 92, 112, 113, 123, 137]; d) mating systems [126, 131, 132, 133, 151]; e) introgression and hybridization [16, 72, 89, 113, 125]; f) cultivar and species identification [91, 153]; among others.

Most of these studies involve terrestrial orchid species, of the genera *Anacamptis* Rich. [39], *Caladenia* R. Br. [100], *Calopogon* [137], *Calypso* Salisb. [6], *Cephalanthera* Rich. [21, 36, 114], *Cleistes* [123], *Cremastra* Lindl. [36], *Cymbidium* [33, 34, 91, 92, 93], *Cypripedium* L. [1, 18, 22, 29, 30, 72, 149], *Dactylorhiza* [24, 63, 101], *Diuris* Sm. [116], *Epipactis*



Zinn. [23, 43, 61, 66, 112, 126, 127], *Eulophia* [133], *Goodyera* [69, 151]; *Gymnadenia* [55, 56, 57, 111, 124], *Gymnadenia* (syn. in ref.: *Nigritella*) Rich. [64], *Listera* [20], *Ophrys* [26, 125], *Orchis* Tourn. ex L. [8, 9, 108, 113], *Platanthera* [146, 147, 148], *Pseudorchis* Seg. [104], *Pterostylis* R. Br. [117, 118], *Serapias* L. [102], *Spiranthes* [10, 46, 83, 130, 131, 132, 135], *Tipularia* Nutt. [122], *Vanilla* Plum. ex Mill. [16, 89, 90], *Zeuxine* [132, 133], among others.

Although most of the orchid species (about 73%) are epiphytes [11, 42], population biology studies with molecular markers in epiphytic and lithophytic species are modest compared to terrestrial orchids, including the genera: *Catasetum* Rich. ex Kunth [87], *Dendrobium* [153], *Laelia* [105, 138], *Lepanthes* Sw. [139], *Oncidium* (syn. in ref.: *Tolumnia* Rafinesque) [3], *Pleurothallis* [17], *Miltonia* Lindl. and *Prosthechea* Knowles & Westc. [Veasey EA et al. unpublished data].

## Population Biology Studies in Terrestrial Orchids

*Orchis* is the largest European genus, of which 70 terrestrial species are distributed in Europe, the Middle East, north Africa, and temperate regions of Asia from the Himalayas to Japan. The species are extremely diverse in appearance due to wide and varying habitat. To assess the genetic variability among and within species and the phylogenetic relationships within this group, 31 populations of 11 species of the genus *Orchis* were evaluated [113]. Compared with other outbreeding species ( $H_e=0.086$ ;  $\bar{P}=37.0\%$ ), high heterozygosity values were obtained for *Orchis* species ( $H_e=0.149$ ;  $\bar{P}=43.9\%$ ) [51]. A low  $G_{ST}$  mean value (0.070) was also observed, practically equal to the one reported for outbreeders (0.071) [81]. Further studies with the species *O. papilionacea* L. and *O. morio* L. [8, 108], have found low interpopulation variation with high levels of gene flow ( $N_m = 5.9$ ) [8] among populations.

High level of genetic variability was also reported for the outcrossing species *Epipactis helleborine* L. Crantz ( $H_e=0.233$ ;  $\bar{P}=59.0\%$ ) [112], ( $H_e=0.150$ ;  $\bar{P}=33.2\%$ ) [66], ( $H_e=0.274$ ;  $\bar{P}=73.6\%$ ) [43], ( $H_e=0.294$ ;  $\bar{P}=67.0\%$ ) [126], ( $H_e=0.058$ – $0.164$ ;  $\bar{P}=18.2$ – $40.9\%$ ) [23]. Low interpopulation variation was observed for this species as well ( $G_{ST}=0.033$ ) [112], ( $F_{ST}=0.134$ ) for rural populations [66], ( $F_{ST}=0.090$ ) [43], ( $F_{ST}=0.040$ – $0.130$ ) [23]. Considering the distribution of genetic variability, the majority of the variation was partitioned within

populations (96.7%, 91.34%, 72.19%, 76.30%, respectively) [23, 43, 112, 126].

A comparison between populations of *E. helleborine*, introduced in the United States and Europe [126], does not indicate a genetic bottleneck associated with the introduction process. Higher levels of intrapopulation genetic diversity and lower levels of interpopulation differentiation in introduced relative to European native populations were obtained with allozymes and cpDNA RFLPs. Also, an extremely low pollen to seed flow ratio (1.43 : 1) was observed for this species [126], although orchid seeds are considered to play a more important role than pollen in gene flow between populations, with seed dispersal having more influence on the genetic structure in orchid species [99].

*E. palustris* L. Crantz has also shown high levels of genetic variability ( $H_e=0.085$ ;  $\bar{P}=29.0\%$ ), comparable to the mean value reported for outbreeders [51], whereas *E. microphylla* (Ehrh.) Sw. was monomorphic for all the loci examined in the two populations studied [112], which may be attributed to the fact that the populations originated from the same genetically impoverished ancestral population. The orchid, *E. helleborine* subspecies *helleborine* (syn. in ref.: *E. youngiana* A. J. Richards & A. F. Porter [106]), growing on zinc- and lead-rich sites in Northumberland, England, is supposed to have originated through the stabilization of a hybrid product via autogamy, after hybridization between *E. helleborine* x *E. leptochila* (Godfery) Godfery or *E. helleborine* x *E. phyllanthes* G. E. Sm. A survey of isozyme variation with 17 loci has ruled out the possibility of *E. phyllanthes* being a possible parent of *E. youngiana* [61]. High levels of genetic diversity ( $H_e=0.11-0.18$ ;  $\bar{P}=40.0-53.0\%$ ) and a genetic structure indicative of outcrossing ( $G_{ST}=0.093$ ) were observed for this species. A hybrid swarm was detected at a Glasgow site [61] for *E. youngiana* with two of its putative parents, *E. helleborine* and *E. leptochila*.

High level of genetic variation was also found in five species of the genus *Calopogon*. There are only about six terrestrial species in this genus distributed in the southeastern United States and one species in eastern Canada. All five species maintained high levels of allozyme variation within their populations ( $H_e=0.11-0.43$ ;  $\bar{P}=50.0-94.4\%$ ). In *C. oklahomensis* D.H.Goldman, *C. pallidus* Chapm. and *C. tuberosus* (L.) Britton, Serns & Poggenb., most of the genetic variation exists within, rather than among populations ( $G_{ST}=0.037-0.085$ ), and *C. multiflorus*

Lindl., which has the most restricted range and rarest occurrence, had the lowest mean genetic diversity values [137].

*Cypripedium* is a predominantly insect-pollinated terrestrial genus in the subfamily Cypridioideae, which contains 30 to 40 species of long-lived, herbaceous perennials ranging from arctic areas to the subtropics of the Northern Hemisphere [82]. Twelve of these species occur in North America [30, 82]. Different genetic variation levels have been reported for *Cypripedium* species. A high level of polymorphism was reported for the American populations of northern *C. parviflorum* var. *pubescens* (Willd.) O. W. Knight and *C. parviflorum* var. *makasin* (Farw.) Sheviak ( $H_e=0.22-0.29$ ;  $\bar{P}=81.8\%$ ) [149], as well as for *C. calceolus* L. 1753 ( $H_e=0.244$ ;  $\bar{P}=75.0\%$ ) [29] and ( $H_e=0.184$ ;  $\bar{P}=45.5\%$ ) [22]. Lower levels of genetic variation were found in *C. reginae* Walter ( $H_e=0.037$ ;  $\bar{P}=18.0\%$ ) [30], *C. acaule* Aiton ( $H_e=0.0016-0.023$ ;  $\bar{P}=5.3-15.4\%$ ) [18], while a total lack of genetic diversity ( $H_e=0.00$ ;  $\bar{P}=0\%$ ) was reported in *C. arietinum* R. Br. in W. T. Aiton populations [18, 30]. The complete lack of genetic variation in this taxon in Michigan at the species level suggests a genetic bottleneck event that has eliminated variation in a population ancestral to the current populations. The same hypothesis is applied to the low genetic diversity observed in *C. reginae* and *C. candidum* Muhl. Ex Willd. populations [30].

Considering the genetic structuring of populations, most of the total species-level variation was found within populations of *C. calceolus* (81%) [29], (98, 4%) [22], *C. acaule* (84%) [30], and *C. candidum* (93%) [30]. These values are in accordance with the results reported previously for *E. helleborine* [23, 43, 112, 126], also an outcrossing orchid species.

Another terrestrial genus, *Spiranthes*, contains about 30 to 50 primarily terrestrial and a few epiphytic or lithophytic species distributed globally. Most species are found in tropical and subtropical regions of the world except Madagascar, tropical America and tropical Africa. *S. spiralis* (L.) Chevall., widely distributed in southern Europe, in the Mediterranean region, and in north Africa, *S. sinensis* (Pers.) Ames and *S. hongkongensis* S.Y.Hu & Barretto, both once widespread in Hong Kong, becoming recently rare, *S. diluvialis* and *S. romanzoffiana*, the latter restricted to the British Isles in Europe, were subjected to population genetic studies [10, 46, 83, 131, 132]. These species have shown contrasting mating systems and genetic diversity characteristics. *S.*

*sinensis*, a pollinator dependent outcrosser [131], and *S. spiralis*, where outcrossing is considered possible, being facilitated by protandry and by sequential flowering within inflorescences [83], have shown considerable levels of genetic variability, with low genetic differentiation among populations of *S. spiralis* ( $F_{ST}=0.022$ ), and *S. sinensis* ( $G_{ST}=0.174$ ), with 83% of the total gene diversity within populations for the latter [83, 131].

A pollinator-independent selfer allotetraploid, *S. hongkongensis*, exhibited almost complete genetic uniformity both within and among populations [131, 132]. According to the author, this result would not be directly related to the autogamy mating system, but to a population bottleneck hypothesis, where chromosome number and gene duplication at many isozyme loci suggest that this species probably evolved through a single hybridization event between diploid *S. sinensis* and *S. spiralis* [130]. Thus, the population bottleneck associated with its origin would lead to genetic uniformity irrespective of its mating system. Another allotetraploid species, *S. diluvialis*, however, exhibited high intrapopulation genetic variability ( $A=2.6-3.3$ ;  $\bar{P}=57.1-71.4.0\%$ ), and a low interpopulation divergence ( $F_{ST} = 0.083$ ) among 12 populations from Utah and Colorado, USA [10]. But a subsequent study evaluating 23 populations of *S. diluvialis* representing its entire geographical range [135], revealed no genetic variation within or among populations through PCR-RFLP analysis of the nuclear ribosomal internal transcribed spacer (ITS) and mitochondrial and chloroplast DNA noncoding regions. DNA sequencing revealed that *S. diluvialis* has rDNA of both *S. magnicamporum* Sheviak. and *S. romanzoffiana*, supporting the proposed origin of the allotetraploid.

Different patterns were observed between northern and southern European populations of *S. romanzoffiana*, when investigated with chloroplast microsatellites and AFLP markers [46], which are in agreement with the contrasting published generalizations that orchids show either higher, or lower, levels of population differentiation than other plant families.

Confirming this, Sharma et al. [117, 118] observed unusually high levels of genetic variability in two terrestrial, restricted in distribution, *Pterostylis* species: *P. aff. picta* M.A. Clem. ( $H_e = 0.284$ ;  $\bar{P}=69.47\%$ ) and *P. gibbosa* R.Br. ( $H_e=0.261$ ;  $\bar{P}=69.0\%$ ). *P. gibbosa* is a rare and endangered Australian orchid, distributed on the Central Coast of New

South Wales with disjunct populations near Milbrodale on the North Coast [117]. Despite the potential to extend its geographical range to adjacent similar habitats as seeds are wind blown, high genetic variability and high seed viability (68-90%), *P. gibbosa* is confined to only four relictual sites. Several hypotheses were raised to explain the high genetic variability of these geographically isolated populations, such as: the outcrossing nature of this species through specialized pollination system, high fecundity, wind dispersal of seeds and high level of gene flow, and strong summer winds which sometimes occur in October-November at the time of seed capsule dehiscence. An alternative hypothesis is that all these populations were derived from a common ancestral stock and later spread to different areas, but still maintaining the similar genetic structure due to similar evolutionary forces and ecological characteristics in these areas [117]. A population differentiation ( $G_{ST} = 0.15$ ) comparable to other species with wind dispersal seeds ( $G_{ST} = 0.14$ ) [81], suggests that the long-distance wind dispersal is an effective means of maintaining gene flow among distant populations. As with other orchids such as *E. helleborine* [112], *Orchis* species [108, 113], *Cypripedium* species [30], *Gymnadenia conopsea* R.Br. in W.T. Aiton [111], *P. aff. picta* [118], most of the variation resides within populations (85%) for *P. sibbosa* [117].

*P. aff. picta*, another endemic Australian *Pterostylis* species, is a small geophyte restricted to southwestern Australia, growing in deep calcareous sand in shrubby forests. This orchid is considered to be endangered and the total wild population is estimated to be less than 250 plants in nine scattered populations [118]. However, the high genetic diversity found in this species contrasts with the notion put forward by Hamrick and Godt [58] that a narrow geographical range should coincide with low genetic variation. Similar hypotheses to those formulated for *P. gibbosa* [117] were considered by the authors [118] to explain the high genetic diversity in *P. aff. picta*.

A different result was obtained with another threatened species, *Platanthera leucophaea* (Nutt.) Lindl., a long-lived, perennial outcrossing pollinated by nocturnal hawkmoths. Wallace [146] found very low levels of diversity ( $H_e = 0.103$ ;  $\bar{P} = 12.0\%$ ) and high levels of population differentiation ( $F_{ST} = 0.75$ ) and high inbreeding coefficients in five of the 10 populations surveyed with allozyme markers, from Michigan and Ohio, USA. In contrast, RAPD markers showed higher levels of

polymorphism ( $P_p = 45.0\%$ ) and moderate measures of population differentiation ( $G_{ST} = 0.26$ ). Genetic and geographic distances were not significantly correlated in this study, suggesting a lack of interpopulation gene flow and/or genetic drift within populations. It is widely believed that *P. leucophaea* speciated from *P. praeclara* Sheviak & M. L. Bowles by adapting to a different suite of pollinators [119]. Furthermore, after the close of the Wisconsinan glaciation, *P. leucophaea* probably rapidly colonized the prairie peninsula around the Great Lakes and invaded an ecologically diverse landscape [146]. Thus, the lack of allelic variation at most loci and fixation of alleles in several populations are consistent with founder events by a small number of individuals and variable source populations. Although it is expected that gene flow was extensive shortly after colonization, recent fragmentation of its habitat and isolation of populations may have increased the allelic differences among populations today [146].

Two other terrestrial orchid species showed low levels of genetic variation with isozyme markers and higher values for RAPD markers. Wong and Sun [151] reported low variation both at the population ( $H_e = 0.073$ ;  $\bar{P} = 21.78\%$  and species ( $H_e = 0.15$ ;  $\bar{P} = 33.0\%$ ) levels, in comparison with other animal-pollinated outbreeding plant species, and higher levels for both population ( $H_e = 0.18$ ;  $\bar{P} = 55.13\%$ ) and species ( $H_e = 0.29$ ;  $\bar{P} = 97.03\%$ ) levels with RAPD markers in *Goodyera procera* (Ker Gawl.) Hook. However, a restricted interpopulational variation was found for both isozymes ( $G_{ST} = 0.52$ ) and RAPD ( $G_{ST} = 0.39$ ) markers, much above the average for outcrossing species, suggesting that gene flow was limited in this species. Another study found a lack of isozyme variation for the species *Zeuxine gracilis* (Breda) Blume, an outcrosser with restricted distribution, *Z. strateumatica* (L.) Schltr., an apomictic colonizer found only in newly available open habitats, and *Eulophia sinensis* Miq., an outcrossing colonizer [133]. Higher genetic variation levels were found at the RAPD loci within populations of *Z. gracilis* ( $H_e = 0.54$ - $0.076$ ;  $\bar{P} = 15.88$ - $21.65\%$ ) and *E. sinensis* ( $H_e = 0.070$ - $0.084$ ;  $\bar{P} = 17.82$ - $20.97\%$ ), but little variation existed within populations of the apomictic *Z. strateumatica* ( $H_e = 0.011$ ;  $\bar{P} = 2.58$ - $2.84\%$ ). Independent of the breeding system, high  $G_{ST}$  values were obtained for all three species, indicating that total gene diversity was partitioned primarily between populations [133].

Molecular markers have also been used in orchids to unravel doubts concerning the origin of allotetraploids. The origin of *Phatanthera huronensis* Lindl., considered an allopolyploid derivative of *P. dilatata* (Pursh.) Lindl. ex L.C.Beck. and *P. aquilonis* Sheviak was confirmed from variation at 305 ISSR and RAPD loci and cpDNA patterns generated from amplification and digestion of two noncoding regions, rp116 intron and *tmT-trnF* region [147]. The genetic structure using ISSR markers of these three species revealed that most of the variation occurs within populations for *P. dilatata* ( $\phi_{ST} = 0.48$ ) and *P. huronensis* ( $\phi_{ST} = 0.36$ ), where  $\phi_{ST}$ , an equivalent measure to  $F_{ST}$  used for dominant markers, is the combined percentage of variation occurring among groups and among populations, while most variation occurs among populations for *P. aquilonis* ( $\phi_{ST} = 0.69$ ) [148]. According to the author, self-pollination via autogamy is a likely cause of the lower level of variation and greater structure observed in *P. aquilonis*. On the other hand, because *P. dilatata* and *P. huronensis* are thought to be primarily outcrossing, gene flow via pollen may be more extensive in these species, reducing therefore differentiation among populations, while promoting variability within populations.

Allozyme data confirmed the origin of the tetraploid *Gymnigritella runei* Teppner & E. Klein, formed by fusion of an unreduced gamete from *Gymnadenia nigra* (L.) Rchb.f. (syn in ref.: *Nigritella nigra* subspecies *nigra* [L.] Rchb.f.) with a normal, haploid gamete from *Gymnadenia conopsea* (L.) [64]. Also in this study, the multilocus genotype found in *Gymnadenia widderi* (Teppner & Klein) Teppner & E. Klein (syn. in ref.: *Nigritella widderi*) was identical to one of the multilocus genotypes found in *Gymnadenia nigra* (syn. in ref.: *N. miniata* [Crantz] Jach.), indicating a close relationship of these taxa.

*Gymnadenia* is a genus distributed in the wet grasslands of northern temperate regions, with about 15 terrestrial species, mostly dwarf alpine orchids. Genetic and floral divergence among sympatric populations of *G. conopsea*, a common orchid in central Europe, were conducted by Soliva and Widmer [124], comparing early-flowering populations in Switzerland, recognized as subspecies *conopsea*, and late-flowering populations, recognized as subspecies *densiflora*, which occur in sympatry but with separate flowering periods. Allozyme variation indicated that subspecies *conopsea* was significantly more variable than subspecies *densiflora* and that gene flow between subspecies was low, suggesting that the difference in flowering phenology represented an effective barrier to



gene flow and may be associated with genetic divergence and taxonomic diversification. Further studies were conducted with *G. conopsea* [55, 56] using microsatellite markers. High genetic variation within, and low genetic divergence among ten Swedish populations of *G. conopsea* were found, although the correlation between population size and number of alleles was close to significance at the 95% level [55]. This species is pollinated by highly mobile butterflies and moths, and presents wind-dispersed seeds, which according to the authors probably counteracts the effects of fragmentation.

Three species of *Cephalanthera*, a genus of 15 herbs, perennial and achlorophyllous species, distributed in the temperate regions of North Africa, southern Himalayas, Japan, Europe and W. North America [107], presenting different breeding types were evaluated: (1) *C. longifolia* (L.) Fritsch, a normal outbreeder, (2) *C. rubra* (L.) Rich., an outbreeder with facultative vegetative reproduction, and (3) *C. damasonium* (Mill.) Druce, an inbreeding species [114]. The last species showed a total lack of both among and within population genetic variation, probably due to the autogamic breeding type according to the authors, also suggested for the self-pollinating *P. aquilonis* [148], *Epipactis dunensis* (T. Stephenson & T.A. Stephenson) Godfrey, *E. leptochila* and *E. muelleri* Godfrey, which were completely homozygous and uniform for the allozyme loci measured [127], in marked contrast to the genetically variable *E. helleborine* [43, 112, 126], as mentioned above, and the outcrossing *C. longifolia* ( $H_e = 0.168$ ;  $F_{ST} = 0.104$ ) [114]. These are all examples of how the breeding system in plants can affect their patterns of population genetic variation. The total lack of allozyme variation in these autogamous species may be due to a short time elapsing since one or a few isolated genetically depauperated plants gave origin to a new taxon displaying an autogamous breeding type, where neither mutational events nor natural selection nor drift occurred to alter this homogeneity [114].

Three *C. rubra* populations localized on neighboring mineral islands in the Biebrza National Park, in northeast Poland, were examined with 16 allozyme loci [21], with similar results to those reported for this species [114]. The relatively low levels of genetic variation and clonal diversity in *C. rubra* are mainly a result of the small population sizes, breeding system and type of reproduction, as well as habitat condition. Geographical proximity may be the cause of low genetic differentiation among populations, because it enables gene flow [21]. Two populations



of *C. longibracteata* Blume, a self-compatible, mixed-mating species, exhibited low levels of genetic diversity ( $H_e = 0.036$ ;  $\bar{P} = 18.0\%$ ) and a significant excess of homozygosity ( $F_{IS} = 0.330$ ), consistent with substantial inbreeding via selfing and/or mating among close relatives in a spatially structured population [35]. Spatial autocorrelation analysis revealed moderate but significant local spatial structure in populations of *C. longibracteata*.

In *Calypso bulbosa* (L.) Oakes, bumblebee-pollinated orchid, self-fertilization and substructuring within sampling units may have contributed to the high inbreeding coefficients observed in many *C. bulbosa* populations ( $F_{IS} = 0.283$ ), over all loci within populations, and the long-distance seed and pollen dispersal may have accounted for the low to moderate genetic differentiation among populations [6].

Low genetic variation ( $H_o = 0.058$ ;  $\bar{P} = 9.4\%$ ;  $\bar{A} = 1.09$ ) associated with small population sizes and genetic drift was observed for *Listera ovata* (L.) R.Br. in W.T. Aiton, localized on mineral islands in the Biebrza National Park of northeast Poland, using 32 allozyme loci [20]. Low allozymic genetic variation within-taxon and within-population in Scandinavian *Pseudorchis albida* (L.) Á.Löve & D.Löve ( $\bar{P} = 6.7\%$ ;  $\bar{A} = 2.0$ ) and *P. albida straminea* (Fernald) Soják ( $\bar{P} = 16.7\%$ ;  $\bar{A} = 2.0$ ) was attributed to ancient founder events [104]. According to the authors, although the differentiation is small, present-day distributions of taxa suggest that the divergence probably started before the Weichselian glaciation period.

Effective population size ( $N_e$ ) influences the degree to which random genetic drift changes allele frequencies, increases inbreeding, and decreases genetic diversity, and thus is a parameter of direct relevance to the conservation of rare species [36]. Using isozyme markers and spatial autocorrelation analysis, six populations of the rare *Cremastra appendiculata* (D.Don) Makino were studied in a large (180 ha), undisturbed landscape on Oenaro Island, South Korea [36]. The levels of genetic variation ( $\bar{P} = 34\%$ ;  $\bar{A} = 1.40$ ;  $H_e = 0.122$ ) observed were greater than expected, given the estimates of  $N_e$ , leading the authors to consider historical factors resulting in  $N_e$  being greater in the past than in present-day populations. Spatial autocorrelation analyses indicated significant fine-scale genetic structure, suggesting positive spatial aggregation of clones (clonal structure) and the presence of related genets (family structure) within *C. appendiculata* populations.

A fine-scale population genetic structure was also examined for the terrestrial *Cymbidium goeringii* (Rchb.f) Rchb.f in W.G. Walpers, a small herbaceous perennial, using spatial autocorrelation statistics [34]. All visible individuals (138 and 110, respectively) within 20 x 40 m areas of each of two populations were sampled and their locations mapped. Fourteen allozyme loci were analyzed and Moran's spatial autocorrelation statistics were calculated for a large number of alleles. Results indicated that genetic similarity was shared among individuals within up to a scale of 14 m distance, which was partly due to a combination of limited pollen dispersal and long-distance seed dispersal by wind. The authors recommend, therefore, that sampling within populations should be conducted at 14-16 m intervals [34]. Another study investigated the levels of genetic diversity of 24 populations of *C. goeringii* from Korea and Japan, revealing high levels of genetic variation both at population ( $H_e = 0.238$ ) and species levels ( $H_e = 0.260$ ) using 14 allozyme loci [33]. High values of gene flow ( $N_m=2.06$ ), based on  $G_{ST}$  values, were estimated, suggesting that genetic drift is not a major fact in these populations. A significant correlation between geographic distance and genetic distance was found in *C. goeringii*. However, a relatively low interpopulation variation value was observed ( $G_{ST}=0.029$ ), even though the land connection between the southern Korean peninsula and southern Japanese archipelagos has not existed since the middle Pleistocene [33]. These data are suggestive of the potential for long-distance seed dispersal expected in the Orchidaceae [3], with large numbers of small seeds of *C. goeringii* probably traveling long distances by wind from populations both in Korea and Japan.

A few orchids, such as *Ophrys* species, are pollinated by male bees and wasps through sexual deception. *Ophrys* is a species-rich genus with over 140 species, centered on the coast of the Mediterranean Basin [125]. Sympatric populations representing different species of the *O. sphegodes* Mill. complex, that differ slightly in floral morphology and are pollinated by different solitary bee species, were studied using microsatellites to test whether gene flow across the species boundaries occurs in these sympatric populations, or whether they are reproductively isolated [125]. The authors observed introgression between co-flowering, sympatric populations, therefore contradicting the assumption that species boundaries in *Ophrys* and other sexual deceit pollinated orchids

are maintained by strong pollinator specificity [77]. This specificity is achieved through the mimicking of female insects with pollinator-specific odor signals. But in this study gene flow among conspecific *Ophrys* populations is high over short geographic distances and may be the consequence of either pollen flow through pollinator movement, indicating that pollinators occasionally perform heterospecific pollination, or passive seed dispersal [125]. Also observed was an extensive genetic diversity, suggesting that drift plays a minor role in the evolution of this orchid lineage. Another study with *Ophrys* species was conducted with RAPD analysis, evaluating four allopatric populations of the *O. bertolonii* Moretti complex, as well as two populations of *O. bertolonii* s.str. Moretti and *O. sphegodes* subspecies *sphegodes* (syn. in ref.: *O. fuciflora* Curtis), with significant gene diversity observed for all six taxa, suggesting their separation at specific (or subspecific) level [26].

*Caladenia tentaculata* Schltdl. is also a deceptive orchid species. It possesses both food deceptive and sexually deceptive species. Allozyme analysis was conducted in order to verify if the pollinator behavior is likely to result in outcrossing and long-distance pollen flow in *C. tentaculata* populations [100]. Outcrossing and long-distance pollen flow were confirmed in this study, where the maximum pollen flow distance observed was 58 m. Thus, the authors concluded that deceptive pollination in this species results in long-distance pollen flow. As far as the genetic variability parameters were concerned ( $\bar{A}=1.6$ ;  $\bar{P}=24.2$  ;  $H_e=0.091$ ), the mean number of alleles per locus was above average, while  $P$  and  $H_e$  were a little below average for animal pollinated outcrossing species, but close to typical values for mixed-mating plants [58]. Within-population fixation indices ( $F$ ) close to zero and an unusually low interpopulation differentiation  $\theta = 0.034$  ( $\theta=F_{ST}$ ) were observed for *C. tentaculata* [100], in accordance with its outcrossing mating system.

Two different pollination strategies are known for North American *Cleistes* [123], a genus comprising around 56 terrestrial orchid species distributed throughout the Americas, from eastern North America south to Brazil [40]. Its flowers can act as 'food-fraud' mimics [4], where the yellow labellar crest of the nectarless and scentless flower of West Virginia *C. bifaria* (Fernald) Catling & Gregg probably mimics pollen, thus attracting naive bees seeking food, or they can present a reward strategy [53]. For instance, at the Brunswick County savannah in coastal

North Carolina, where a substantial proportion of bumblebee pollinators collect pollen. Flowers of *C. bifaria* emit a strong vanilla scent, whereas those of *C. divaricata* (L.) Ames produce a daffodil-like scent. In this case, floral fragrance is thus associated with pollen reward and may encourage bees to visit the flowers [123]. To test whether the development of different fragrances, as well as presenting peak flowering times one week apart [31], is a possible instance of character displacement and an evidence for selection against hybrid formation, molecular analyses using AFLP, DNA sequencing, and microsatellites, corroborated the absence of gene flow where the two taxa occur sympatrically [123]. In the same study, genetic links occurred between the coastal plain *C. divaricata* and the two mountain populations of *C. bifaria*. This fact raised the question whether gene flow might be occurring among these groups. This possibility was ruled out because of the long distances between the two areas. The most accepted hypothesis was of a recent common ancestor as opposed to contemporary gene flow [123].

A different result was reported for the genus *Vanilla*, composed of 100 terrestrial or hemiepiphytic species, distributed in tropical and subtropical regions of North America, Mexico, West Indies, Central America, South America, Africa, Southeast Asia and West Pacific Islands [107]. The occurrence of natural hybrids between sympatric *V. claviculata* (Sw.) Sw. and *V. barbellata* Rchb.f. species were confirmed using morphological, isoenzymatic and pollination experimental data [89]. A significant surplus of heterozygotes in the deviating individuals corroborates the theory that the individuals were  $F_1$  hybrids, and not backcrosses or  $F_2$  generation individuals, which would result from introgression. Post-pollination barriers that could separate both parental species were ruled out after interspecific artificial crossings were made. However, hybrids were only discovered in the area where the two parentals come into contact, where the two species have almost synchronous flowering times and are likely to share the same pollinator. Also, *V. claviculata* was typically found in moist serpentine forests, while *V. barbellata* seemed to exploit dryer habitats, so hybridization is presumably normally avoided by the prezygotic mechanism of spatial isolation because of minor differences in habitat preference [89].

RAPD markers were used to assess the levels of genetic diversity in cultivated *V. planifolia* Jacks ex Andrews in introduced areas of Reunion

Island (Indian Ocean), the relationships between *V. planifolia* (including the now invalid *V. tahitensis* J.W.Moore) and *V. pompona* Schiede representatives, and the legitimacy of putative *V. planifolia* x *V. tahitensis* hybrids [16]. Low levels of genetic diversity were detected in *V. planifolia*, which is in accordance with the vegetative mode of reproduction of vanilla, and the history of recent introduction in these regions. Based on the RAPD data, the authors suggested that *V. tahitensis* is probably not a species of direct hybrid origin (*V. planifolia* x *V. pompona*) but rather a species related to *V. planifolia*. *V. tahitensis* was eventually included in *V. planifolia* ([www.kew.org/monocotChecklist](http://www.kew.org/monocotChecklist)).

## Population Biology Studies in Epiphytic and Lithophytic Orchids

The vast majority of epiphytic orchids is animal-pollinated and has tiny wind-dispersed seeds [41]. Epiphytic plants differ from the terrestrial orchids by being distributed in three dimensions, which results in an individual plant surrounded by more individuals than would be possible in two dimensions. This characteristic can affect the fine-scale genetic structure of their populations, that is, the nonrandom distribution of genetically similar individuals within populations, which can, in turn, influence mating patterns and other population phenomena [138]. With the aim of elucidating whether individual clusters in the same tree contain more than one genotype and what is the spatial distribution and fine-scale genetic structure of genotypes within a population, three large populations of *Laelia rubescens* Lindl. were sampled in the Costa Rican seasonal dry forest [138]. Isozyme analysis revealed high levels of genetic diversity at the population level ( $\bar{A}=2.13$ ;  $\bar{P}=83.3\%$ ;  $H_e=0.199$ ) and low among-population variation ( $G_{ST}=0.016$ ). Also, multiple genotypes within a cluster were observed. These multiple genotypes were not the result of somatic mutation within a single individual, but perhaps represented the deposition and preferential establishment of sexually derived progeny in existing clusters [138].

Spatial and temporal genetic structures were also examined across sites on islands and mainlands (continuous forest area) of *Catasetum viridiflavum* Hook. using 17 polymorphic allozyme loci [87]. High levels of allelic diversity were obtained, with a total of 94 alleles detected for 17 polymorphic loci. Low among-population differentiation was observed across the landscape suggesting that the species-specific pollinator and

tiny wind-dispersed seeds maintain interconnections among distant patches. This level of differentiation is within the range reported for wind dispersed, long-lived perennial, and endemic species [59]. The tiny orchid seeds are likely to traverse distances of 100 m to > 1 km depending on the height of seed release and local wind speed [87].

Another study made with *Pleurothallis*, a large genus of about 2,500 epiphytic species distributed in the tropics [107], occurring in the Brazilian campo rupestre vegetation in the southeastern and northeastern regions of Brazil, mainly in Minas Gerais and Bahia states, found surprisingly high genetic variation levels ( $\bar{A}=2.1-3.8$ ;  $\bar{P}=58-83\%$ ;  $H_e=0.25-0.42$ ) in all five species ((1) *P. johannensis* Barb.Rodri., (2) *P. teres* Lindl., (3) *P. ochreatea* Lindl., (4) *P. fabiobarrosii* Borba & Semir, and (5) *P. adamantinensis* Brade), in spite of the fact that the five species are pollinated by small flies whose behavior enables self-pollination [17]. The authors suggest that self-incompatibility, inbreeding depression, and mechanical barriers that prevent self-pollination in these species are responsible for maintaining such high genetic variability. Moderately low values of  $F_{ST}$ , interpreted as a low level of genetic structuring, were found in *P. johannensis*, *P. fabiobarrosii* and *P. adamantinensis*. The other two species showed higher interpopulation differentiation ( $F_{ST}=0.21$ ) and ( $F_{ST}=0.17$ ) [17].

Seven Brazilian populations of the epiphytic *Prosthechea calamaria* (Lindl.) W.E. Higgins, which originated in the States of São Paulo, Rio de Janeiro and Espírito Santo, were analyzed with six isozyme loci, showing high genetic variation levels ( $\bar{A}=1.50-2.50$ ;  $\bar{P}=33.3-100\%$ ;  $H_e=0.19-0.46$ ) (Veasey et al. unpublished data). A moderate interpopulation variation ( $H_T=0.43$ ;  $D_{ST}=0.07$ ;  $G_{ST}=0.15$ ) was found, with most of the genetic variation occurring within populations.

High levels of genetic variation ( $\bar{A}=1.43$ ;  $\bar{P}=71\%$ ;  $H_e=0.21$ ) were found in *Oncidium variegatum* Sw (syn. in ref.: *Tolumnia variegata* (Sw.) Braem), a widespread, morphologically variable, twig epiphyte of the Caribbean that frequently occurs in large populations [3]. Nearly all genetic variation occurred within populations ( $H_T=0.22$ ;  $D_{ST}=0.03$ ;  $G_{ST}=0.11$ ), with moderate average gene flow estimated among populations. Comparing mainland and island populations, genetic differentiation was more substantial among islands. A significant negative correlation was observed between geographic distance and either genetic

identity or  $Nm_w$  (gene flow based on Wright's statistics [152] among populations.

The potential role of genetic drift in orchid populations was investigated by estimating effective population sizes ( $N_e$ ) in three Puerto Rican species of *Lepanthes*, one of the most species-rich genera in the family [139]. The three species investigated are: (1) *L. rupestris* Stimson, a common lithophyte along riverbeds of the northwestern slopes of the Luquillo Mountains, (2) *L. eltoroensis* Stimson 1970, an epiphyte restricted to mountain ridges along El Toro and Tradewinds trails and Cerro El Cacique in the Caribbean National Forest, and (3) *L. rubripetala* Stimson, a rare epiphyte in Puerto Rico. All estimates of  $N_e$  were usually <40% of the standing population size, resulting in values of <20 individuals per population. Restricted gene flow among populations was observed with isozyme analysis, in the range of one or less successful migrant per generation. This result led to another not surprising inference of high genetic differentiation among populations. Therefore, it was concluded that genetic drift is likely to be important for population differentiation in *Lepanthes* as a result of small effective population sizes and restricted gene flow [139].

Molecular markers such as AFLP and RAPD have also been used in epiphyte or lithophytic species to identify species or hybrid genotypes and to estimate genetic relationship among these taxa. Forty-three commercial *Dendrobium* hybrids were subjected to AFLP analysis and each hybrid tested had a distinct AFLP fingerprint profile, except the tissue culture mutants [153]. AFLP fingerprint profiles were uniform in different parts of tested plants, stable among individuals in vegetative propagated populations throughout different growth periods, showing potential to be an integral part of current new plant varieties protection systems. RAPD marker was used to evaluate 24 accessions of subtribe Oncidiinae, of approximately 1,000 species classified into 56-78 genera, including *Oncidium*, *Odontoglossum* Kunth in F.W.H von Humboldt, *Miltonia* Lindl., *Miltoniopsis* God.-Leb., *Brassia* R.Br. in W.T.Aiton, *Ada* Lindl., *Gomesa* R.Br. and *Comparettia* Poepp. & Endl., most commonly used as cross parents [141]. Fourteen primers produced 263 bands, of which 257 revealed polymorphism. Cluster analysis showed six major clusters and one individual not belonging to any of those. The genus *Miltonia* was segregated from other genera among the Oncidiinae in this study.



With the objective of investigating the level of genetic variability among populations of *Miltonia spectabilis* Lindl. and *M. flavescens* (Lindl.) Lindl., patterns of population variability of *M. spectabilis* were compared with the population of *M. spectabilis* var. *moreliana*. Based on these results, the taxonomic status of *M. spectabilis* var. *moreliana* was evaluated and seven isozyme loci were analyzed (Veasey et al. unpublished data). High diversity levels were observed for *M. spectabilis* ( $\bar{A}=1.43\text{--}2.43$ ;  $\bar{P}=28.6\text{--}71.4\%$ ;  $H_e=0.13\text{--}0.28$ ) and *M. spectabilis* var. *moreliana* ( $\bar{A}=2.14$ ;  $\bar{P}=85.7\%$ ;  $H_e=0.35$ ). Lower values were obtained for *M. flavescens* ( $\bar{A}=1.14\text{--}1.43$ ;  $\bar{P}=14.3\text{--}42.9\%$ ;  $H_e=0.04\text{--}0.16$ ). A cluster analysis showed a clear separation between both species, but did not separate *M. spectabilis* var. *moreliana* from the other populations of *M. spectabilis*, which is not in accordance with a morphometric analysis of floral characters showing a clear distinction of the two taxa [27]. Based on morphometric data, the authors proposed an old species name, *M. moreliana*, whose assumption was not supported by the isozyme data. Officially, *Miltonia spectabilis* var. *moreliana* is now a synonym of *M. moreliana* A.Rich ([www.kew.org](http://www.kew.org)).

## CONCLUSION

There are many research groups in the world tackling the genome evolution of orchids. However, the bewildering size of this family calls for an immense amount of work yet to be done. Among the relatively simple things that should be done is basic cytogenetics. The confusion between the effects of euploidy and Robertsonian aneuploidy may be alleviated by studying the karyotype structure. A trade-off between metacentrics and twice as much telocentrics is a good hint in favor of centric fission/fusion. More complex molecular cytogenetic methods, such as FISH (fluorescence in situ hybridization), and molecular genetic methods, such as the comparison of saturated physical maps, or even whole genome sequencing of critically chosen species, should be used to ascertain homologies among chromosomes of different species and test polyploidization hypotheses. Such an approach would help resolve one of the major problems in orchid genome evolution, viz. the relative importance of euploidy and aneuploidy in the production of current karyotypes.



Considering the pervasiveness of polyploidy, and notably allopolyploidy [128], it is noteworthy the relative scarcity of studies using any genetic methods whatsoever in the identification of extant closest relatives of the parents of allopolyploids. It should be very profitable for evolutionary studies involving allopolyploidy to focus on this subject under the point of view of genomic groups. This would involve the systematic observation of meiotic pairing in lattice-like crossings among all species in a genus, but would allow the compartmentalization of the species into gene pool complexes [60] within which gene flow is easy.

Most of the molecular phylogenetic work carried out to date on orchids has been resorted to the ITS and chloroplast regions. Our knowledge about this family would greatly benefit from the extension of the molecular methods to include single- or few-copy nuclear genes and their nontranslated regions for studies at the infrageneric and infraspecific levels, as well as for the use of the coalescent theory in microevolutionary investigations [45] which require a higher level of variability and sampling.

Some deficiencies in the array of techniques employed and some bias in the choice of taxa (chiefly genera or below) included in phylogenetic and genetic studies can be noted. DNA quantification, for instance, is lacking for many species. Most of the genus- and species-level genetic research is done on temperate groups (Orchidinae and Spiranthinae are good examples). This bias is still more pronounced in studies involving ecology. Table 1 may be used as a non-exhaustive survey on the distribution of research over a phylogenetic frame and can aid in enhancing efforts in this area.

The Orchidaceae is also an excellent group for studying microevolutionary processes, such as genetic drift, hybridization, natural selection, gene flow, migration, etc. Most of the population biology studies concerned with such processes using molecular markers reviewed here have been undertaken with terrestrial orchid species (88.7%), while only 11.3% were conducted with epiphytic or lithophytic species, although epiphytic species outnumber the terrestrial species by 73% [11]. Most of these studies have been conducted with temperate species, which indicate that further attention should be given to tropical species, experiencing different environmental conditions. Considering the molecular markers applied in these studies, 60% used isozyme markers, 10% RAPD, 7.5% SSR, 5% IRRS, 3.7% AFLP and 13.8% others (plastid

microsatellites, chloroplast minisatellites, cpDNA RFLP, cpDNA single stranded conformation polymorphism–SSCP, DNA sequencing). Therefore, isozymes are still a powerful tool for genetic population and population biology studies in orchids, although other types of molecular markers have gradually been incorporated to the methodology of recent research.

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## Karyological Evolution of the Genus *Luzula* DC. (Juncaceae)

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### ABSTRACT

The genus *Luzula* DC. (Juncaceae) possesses the very specific cytological characters of: diffuse centromere, post-reductional meiosis, and variation in the chromosome number by agmatoploidy (chromosome fragmentation), or by symploidy (chromosome fusion), as well as by polyploidy. Agmatoploidy can occur as an exclusive mechanism within a taxon, being total or partial (agmatodysploidy), as demonstrated by the *L. spicata* complex ( $2n=12$  12AL, 24 24BL, 14 10AL+4BL, 16 8AL+8BL, 18 6AL+12BL) and the sub-genus *Pterodes*. However, it can also be present combined with polyploidy (*L. alpina*,  $2n=36$  12AL+ 24BL). The best example of karyotype produced by symploidy is  $2n=6A_0L$  of *L. elegans* (sub-genus *Marlenia*). The sub-genus *Luzula* seems to be the only one representing polyploid taxa in the genus. In spite of the presence of these phenomena, which in the genus *Luzula* can be considered to be evolutionary, the chromosome variation is not generalized, the diploid valence ( $2n=12$ , 12AL) being the most widespread in the *Luzulas*. Consequently,  $x=6$  (6AL) can be considered to be the base chromosome number, which forms the starting point from which all chromosomal evolution in the genus *Luzula* can be explained.

**Key Words:** *Luzula*, agmatoploidy, symploidy, diffuse centromere, agmatodysploidy

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## INTRODUCTION

The genus *Luzula* DC. belongs to the family Juncaceae (Liliopsida, Monocotyledons). This family comprises seven genera: *Juncus*, *Luzula*, *Oxychloë*, *Distichia*, *Patosia*, *Marsippospermum* and *Rostkovia*, which contain over 440 species. Within Juncaceae, greatest diversity of species is found in temperate and cold areas of the world. Paradoxically, neotropical regions provide a habitat for the largest number of genera. *Luzula*, with 115 species, and *Juncus* L., with more than 315 species, are the largest genera and the most cosmopolitan, although they show a marked preference for the northern hemisphere. The other genera in the family are composed of one to six species and are concentrated in the southern hemisphere, principally in the southern Andes. The genera *Oxychloë* Phil. (five species), *Distichia* Nees and Meyen (three species) and *Patosia* Buchenau (one species) are endemic in South America; the genera *Marsippospermum* Desv. (four species) and *Rostkovia* Desv. (two species) have been observed in Patagonia, in the Falkland Islands and in New Zealand, the genus *Rostkovia* also grows in the Equator and in certain islands in the South Atlantic Ocean.

The genus *Luzula* is formed of herbaceous plants, perennial (with the exception of *Luzula elegans* Lowe), rhizomatous or stoloniferous, and characterized by hairy sided leaves. According to the new classification [93] the genus is subdivided into three sub-genera: (1) *Luzula* (with seven sections: (i) *Alpinae*, (ii) *Anthelaea*, (iii) *Atlanticae*, (iv) *Diprophyllatae*, (v) *Luzula*, (vi) *Nodulosae*, and (vii) *Thyrsanochlamydeae*), (2) *Marlenia*, and (3) *Pterodes*. The *Luzulas*, almost cosmopolitan, are present in the various stages of vegetation and prefer acidophile or silicicolous environments. They are principally distributed in moderate and even cold climates, while the genus *Luzula* is almost absent in tropical regions. The centres of maximum diversity are the south-west of Europe, the Far East, western North America, South America (the Andes), Australia and New Zealand.

## CYTOLOGICAL CHARACTER OF THE GENUS *LUZULA*

Due to its particular cytological character, the genus *Luzula* was frequently studied in the 1940s and 1950s. This body of work

contributed to better understanding of its karyological variability and evolution. Malheiros and Castro [62], in their karyological analysis of *Luzula purpurea* Link [in Buch] ex E. Mey (*L. elegans* Lowe), for the first time in 1947, signalled the original nature of the chromosomes in the genus: two centromeres for each chromosome, located in each extremity. In the same year, Malheiros and Gardé [64] showed that the doubling of the number of chromosomes is concomitant with a reduction of about half the chromosome length in *Luzulas* and concluded that the chromosome fragmentation is the result of an evolutionary process in the genus; whereas, on the other hand, it was estimated that the chromosomes have a non-localized centromere. Malheiros, Castro and Cámara [63] observed a particular process in the chromosomal cleavage of *L. purpurea* during the two divisions occurring in the meiosis. The divisions are reversed: the first is equational (separation of the sister chromatids), and the second is reductional (separation of the homologous chromatids). During metaphase I, the bivalents form two superimposed rings parallel to the equatorial plane (chromosomal auto-orientation [5, 22, 45, 46, 75]). In metaphase II, each ring, formed of two homologous chromatids, is oriented perpendicular to the equatorial plane (co-orientation). After the meiosis, the tetraspores are not separated and they undergo two successive and synchronised mitotic divisions in each tetrad. The divisions linked to the development of the gametophyte occur before the dehiscence of the anther [31]. In their analysis of chromosomal behaviour of *L. purpurea* under the effects of X-rays, Castro, Cámara and Malheiros [10] confirm the lack of a localized centromere in the *Luzulas*. All of the original chromosomal fragments resulting from irradiation divide independently and are conserved from generation to generation. In 1950, Malheiros-Gardé [65] did the same and showed that the chromosome fragments were inherited from one generation to another in the same way as the polyploid chromosomes, the fragmentation having been caused by an antimitotic agent, morphine. Later, by using colchicine or X-rays, several studies [12, 13, 69] have again demonstrated that the fragmentation of the chromosomes plays a very important role in the evolutionary process of the genus *Luzula* because the fragments experimentally produced are conserved from generation to generation. Braselton [9], in studying the activity of the kinetochores during *Luzula* cell division, concluded that the chromosomes are polycentric, while Godward [36] remarked on the very

unusual mechanism of the chromosomes with diffuse centromere: holocentric or holokinetic chromosomes [7].

From the karyological study of various species of the genus *Luzula*, Nordenskiöld [73] observed that not only the number but also the size of the chromosomes could vary from one species to another. In fact, the chromosome size decreases when they increase in number: (a)  $4\text{--}6\mu$  in length and  $\pm 1\mu$  in width for *L. purpurea* ( $2n=6$ ); (b)  $1.9\mu$  for *L. sylvatica* (Huds.) Gaudin ( $2n=12$ ), *L. luzuloides* (Lam.) Dandy and E. Willm. ( $2n=12$ ) and *L. nivea* (Nathh.) DC. ( $2n=12$ ); (c)  $1.1\mu$ , which is the usual dimension in the genus, for *L. campestris* (L.) DC. ( $2n=12$ ), *L. multiflora* (Ehrh.) Lej. ( $2n=36$ ), *L. frigida* s.l. ( $2n=36$ ), *L. arctica* Blytt ( $2n=24$ ) and *L. parviflora* (Ehrh.) Desv. ( $2n=24$ ); (d)  $0.7\mu$  for *L. spicata* s.l. ( $2n=24$ ); (e)  $0.3\text{--}0.4\mu$  for *L. pilosa* (L.) Willd. ( $2n=c.70$ ) and *L. sudetica* (Willd.) Schult. ( $2n=48$ ). This difference in chromosomal size led to the definition of a specific nomenclature for the karyotypes of the genus *Luzula* by Nordenskiöld as detailed below: in 1951 [74] (1) **AL**: standard chromosome type present in the larger part of species at  $2n=12$  and in the euploid series  $2n=24, 36, 48$  resulting from polyploidy; (2) **BL**: for chromosomes whose size is less than half that of type AL; (3) **CL**: for chromosomes whose length is less than half that of type BL and a quarter of type AL cells. Malheiros-Gardé and Gardé [67] complete this nomenclature by attributing the type **A<sub>0</sub>L** to the *L. purpurea* chromosomes, the only species to possess  $2n=6$  and more than twice as many AL chromosomes. In quoting the work of Portuguese authors, Nordenskiöld (l.c.) admits that the fragmentation may play a very important part in the evolution of the genus, processes linked to the non-localized centromere and to the inverted meiosis. She also demonstrated chromosomal fragmentation in a study of chromosomal behaviour during meiosis in experimental hybrids, which resulted from species possessing different types of chromosomes: *L. campestris*  $2n=12$  (12AL) was crossed with *L. sudetica*  $2n=48$  (48CL), the hybrid possesses  $2n=30$  (6AL+24CL), and in meiosis the AL chromosomes never pair between them, but usually pair with some CL chromosomes.

Several authors discuss the phenomenon of the increasing chromosome number without change in the amount of nuclear DNA: “endo-nuclear polyploidy” Nordenskiöld [73, 74, 75], “pseudo-polyploidy” [6], and “agmato-polyploidy” [37]. Furthermore, the fragmentation cannot occur simultaneously in all the chromosomes. This also explains the intermediate numbers in certain species in the genus

*Luzula*, which results from the process Nordenskiöld (l.c.) named “half-completed endo-nuclear polyploidy”. However, the term nowadays accepted is the one proposed by Malheiros-Gardé and Gardé in 1950 [66]: *agmatoploidy*, from the Greek word *αγμα*, which means rupture, in the multiplication mechanism for the chromosome number linked to the chromosome fragmentation. According to these authors, agmatoploidy is always subordinate to the presence of chromosomes with diffuse centromere. On the other hand, the reverse is not true. In 1964, Ebinger [21] interpreted this phenomenon as follows “...though the chromosome number is doubled, the DNA value remains the same (chromosome ratio 2:1; DNA ratio 1:1). In contrast, in a normal polyploid series, the chromosome number as well as the DNA value is doubled (chromosome ratio 2:1; DNA ratio 2:1)...”

Gardé [34] states that agmatoploidy is the main evolutionary factor in the genus *Luzula*. The results of Nordenskiöld [74] provided Gardé with the proof that the meiosis of chromosomes of different sizes originates from interspecific crossings. However, the cause of agmatoploidy remained unknown to Gardé. Malheiros-Gardé and Gardé [67], who also studied the hybrids and intermediate forms (*L. spicata* s.l., *L. oresteria* Sharsm.), draw the same conclusion. They consider agmatoploidy to be an evolutionary process, but without establishing the causes and the development of the fragmentation. They state that the fragmentation is produced in a median position and that it did not simultaneously intervene strongly in all the chromosomes, which also gives a progressive aspect to the variation of the chromosome number. They call the cytotypes presenting more than one type of chromosome *intermediate forms*. Agmatoploidy has also been defined as “orthoevolution karyotypic”, a process implicating all the chromosomes in the karyotype [101]. In 1952, Noronha-Wagner and Castro [77] studied the meiosis of *L. campestris* and *L. nemorosa* Pollich and E. Meyer. They observed that the meiosis is post-reductional, but the mechanism seemed to be different from that observed in *L. elegans* by Malheiros, Castro and Camara [63]: during the two divisions, the bivalents were placed parallel to the spindle fibres and perpendicular to the equatorial plane, undergoing a break in anaphase I, followed by the fusion of the sister chromatid fragments in telophase I and the homologue chromatids in telophase II. All the same, when the authors analyzed the meiosis in several populations of *L. campestris* [32], no signs of chromosome fragmentation during the two divisions were noticed. Through this



interpretation of meiosis, Noronha-Wagner and Castro, at least in part, provided an understanding of agmatoploidy. During telophase II the fusion of the homologous chromosomes could fail, resulting in an increase in the chromosome number. The fusion could be partial producing intermediate forms, which shows the progressive character of agmatoploidy.

From the 1960s, several studies were dedicated to quantifying DNA using photometry. Mello-Sampayo [70] and Halkka [39], have also either demonstrated or confirmed the hypothesis, according to which the number of chromosomes increases in agmatoploidy without parallel change in the amount of nuclear DNA. Barlow and Nevin [3] suggested that the amount of DNA and the total volume of the chromosomes in the agmatoploid species at 12AL and 24BL are not constant. Nevertheless, their conclusions do not seem to be well founded since their comparison is based on species, which do not belong to the same section, or at least are not akin. In contrast, the authors admit that the karyological evolution of the genus *Luzula* favours the phenomena of fragmentation, creating agmatoploid series of chromosome numbers. More recently, Sen *et al.* [88] analyzed 6 species of *Luzulas* produced by in vitro culture, as Inomata [47] had previously done with *L. elegans*. Their conclusions essentially confirm that the increase in chromosomal number is made by fragmentation, without any variability in the amount of DNA and the chromosomes, with diffuse centromeres. Similar conclusions are drawn in recent the work on the genus *Luzula* [20, 53].

In parallel with agmatoploidy and polyploidy, Noronha-Wagner [76] also consider chromosomal fusion to be a possible auxiliary factor in the chromosomal evolution for the genus *Luzula*, which at first sight would amend the opinion of Stebbins [95], which is: “polyploidy is predominantly an irreversible trend from lower to higher levels. This irreversibility is not due to the genetic impossibility of reversal, but to either the lowered overall adaptability or the evolutionary insignificance of this product”. Stace [94] indicated that the reduction of ploidy level towards diploidy is an extremely rare phenomenon. Concurring with Raven [84], Stace considers all the evolutionary patterns of vascular plants start at diploid level and that the ample spectrum of polyploids finds its origin in the diploids. However, it has been demonstrated that certain vegetable organisms with localized centromere have sustained a reduction in their degree of ploidy: haploidy [16, 48, 87]. On the other

hand, in organisms with diffuse centromere, nothing indicates that fusion is not as probable as fragmentation. From this, Luceño and Guerra [61], implied that chromosomal fusion is another phenomenon permitting chromosomal variability in organisms with holocentric chromosomes. The authors suggest the term *symploidy*, from the  $\sigma\upsilon\nu$  meaning with or together, expressing the idea of chromosomal union. This could explain the reason for such a low number in *L. elegans* ( $2n=6$ ), which may result from chromosome fusion starting from a karyotype at  $2n=12$  [37, 61].

The work on the genus *Luzula* highlights three distinctive cytological characteristics. Firstly, the chromosomes are holocentric because they possess a diffuse or a non-localized centromere. Secondly, the two meiotic divisions are reversed because the equational division, with auto-orientation of the chromosomes, precedes the reductional division (post-reductional meiosis). Thirdly, the variation of chromosome number would result from different processes, that is to say, polyploidy, agmatoploidy (increase in chromosome number by fragmentation or fission of chromosomes, without change in the amount of DNA) and symploidy (decrease in the chromosome number via chromosomal fusion). These characteristics have still not been demonstrated in all the other genera of Juncaceae. According to Bailey (in Stace [94]), the genus *Juncus* L. does not even possess holocentric chromosomes. In fact, these characteristics are rarely widespread in the natural world. Certain works include a list of organisms, which potentially may have holocentric chromosomes [102] without the other two cytological characteristics being present. However, the presence of these chromosomes is reported in very few organisms. In the vegetable kingdom and among unicellular organisms, Geiter [35] was also able to observe reverse meiosis in *Spirogyra* Link (Chlorophyta). Among monocotyledons are the genera *Carex* L. (Cyperaceae) [22, 43, 58] and *Chinographis* (Melanthiaceae) [99], and in dicotyledons *Myristica fragrans* Houtt. (Myristicaceae) [28], the sub-genus *Cuscuta* L. with *C. babylonica* Aucher and Choisy (Convolvulaceae) [79]. Only in the genus *Carex* and in the species *Cuscuta* has it been possible to observe reverse meiosis and the phenomena of chromosomal fusion and fission. In the animal kingdom the presence of holocentric chromosomes has been noted in the following:- (1) phylum Nematoda (*Ascaris* subspecies is and *Caenorhabditis elegans*, [1]). (2) In some arthropods: as in Arachnida of the order Scorpions [89]. (3) In insects of the order Lepidoptera [97] and Hemiptera (Heteroptera, [86]). In addition, holocentric chromosomes

are also accompanied by a reverse meiosis in the order Odonata [78] and in the Coccidae [44] and Aphidae families (Homoptera, Hemiptera [85]).

Castro [11] estimates that chromosomes with non-localized centromere (chromosomes with diffuse centromere or holocentric chromosomes) are more primitive than those with localized centromere, a hypothesis taken up by Vaarama, Halkka, Hughes-Schrader and Schrader, Sybenga (in Wrensch *et al.* [102]), Battaglia [4] and Hakanson [38]. During the course of the evolution, the first may have experienced mutations leading to the localization of the centromere. On the other hand, in the taxa, which have conserved their primitive character, the chromosomes have experienced the phenomenon of fragmentation or fusion engendering new chromosomes without loss of chromosomal material and accompanied by reversed meiotic divisions. From their study of the sub-genus *Cuscuta*, Pazy and Plitmann [79] deduced that because of the rarity of the chromosomes with diffuse centromere in both vegetable and animal kingdoms, this type of chromosomes must be derived and not primitive. This opinion is shared by Greilhuber [37], who states “the diffuse centromere, combined with inverted meiosis, is the only reliable chromosomal higher-level synapomorphy in monocotyledons”.

The larger part of the recent work on the genus *Luzula* does not analyze its karyology closely. Those studies focused on the phylogeny of the genus using a molecular approach relying on sequence analysis of the “internal transcribed spacers” (ITS) of the nuclear ribosomal DNA (nuclear rDNA) (García-Herran, unpublished), or otherwise by analyzing different sequences of chloroplast DNA (cpDNA) [17, 18].

## KARYOLOGY OF THE GENUS *LUZULA*

All available karyological data concerning the genus *Luzula* is shown in Table 1, which summarizes the complete (or entire) existing chromosomal counts in the bibliography relating to each and every taxa belonging to the genus (Species Plantarum [93], site <http://www.unine.ch/caryo/luzula>).

It seems that the chromosome variability in the *Luzulas* is not very wide because the larger part of the species present the diploid valence  $2n=12$  (12AL) [32]. This valence is almost omnipresent in the *Anthelaea*

**Table 1.** Variability of chromosome numbers in the genus *Luzula* DC<sup>x</sup>

Taxon	Diploid chromosome number	Origin of chromosome number
<b>Subg. Marlenia</b>		
<i>L. elegans</i>	2n=6 (6A <sub>0</sub> L)	Symploidy
<b>Subg. Luzula</b>		
Sect. Anthelaeae		
<i>L. seubertii</i>	2n=12	
<i>L. lactea</i> var. <i>lactea</i>	2n=12 (12AL)	
<i>L. canariensis</i>	2n=12	
<i>L. nivea</i>	2n=12 (12AL)	
<i>L. lutea</i>	2n=12 (12AL)	
<i>L. luzuloides</i> s.l.	2n=12 (12AL)	
<i>L. sylvatica</i> s.l.	2n=12 (12AL)	
Sect. Atlanticae		
<i>L. atlantica</i>	2n=12 (12AL)	
Sect. Nodulosae		
<i>L. nodulosa</i>	2n= ?	
Sect. Diprophyllatae	2n=12 (12AL)	
<i>L. wahlenbergii</i>	2n=24 (24BL)	Agmatoploidy
<i>L. piperi</i>	2n=24	
<i>L. hitchcockii</i>	2n=24	
<i>L. glabrata</i>	2n=12	
<i>L. desvauxii</i>	2n=12 (12AL)	
<i>L. parviflora</i> subsp. <i>parviflora</i>	2n=22 (2AL+20BL)	Agmatoploidy+Symploidy
<i>L. parviflora</i> subsp. <i>fastigiata</i>	2n=24 (24BL)	Agmatoploidy
<i>L. parviflora</i> subsp. <i>melanocarpa</i>	2n=24 (24BL)	Agmatoploidy
<i>L. alpinopilosa</i> subsp. <i>alpinopilosa</i>	2n=12 (12AL)	
<i>L. alpinopilosa</i> subsp. <i>obscura</i>	2n=12 (12AL)	
Sect. Alpinae		
<i>L. ulophylla</i>	2n=48 (48CL)	Agmatoploidy
<i>L. traversii</i> var. <i>traversii</i>	2n=46 (46CL)	Agmatoploidy
<i>L. traversii</i> var. <i>tenuis</i>	2n=46 (46CL)	Agmatoploidy
<i>L. celata</i>	2n=12 (12AL)	
<i>L. pindica</i>	2n=24 (24BL)	Agmatoploidy
<i>L. spicata</i> (see the table 2)	2n=12, 14, 16, 18, 24	Agmatoploidy

Table 1 contd.

Table 1 contd.

<i>L. pediformis</i>	2n=12 (12AL)	
<i>L. caespitose</i>	2n=12 (12AL)	
<i>L. alopecurus</i>	2n=24	
<i>L. chilensis</i>	2n=24	
<i>L. racemosa</i>	2n=24	
Sect. <i>Thyrsanochlamydeae</i>		
<i>L. subcongesta</i>	2n=24 (24BL)	Agmatoploidy
<i>L. kjellmaniana</i>	2n=36	
<i>L. arcuata</i> subsp. <i>arcuata</i>	2n=36, 42, 48	
<i>L. arcuata</i> subsp. <i>unalaschkensis</i>	2n=36	
<i>L. confusa</i>	2n=24, 36, 42, 48, 44-48	
<i>L. nivalis</i>	2n=24 (24BL)	Agmatoploidy
Sect. <i>Luzula</i>		
<i>L. pallescens</i>	2n=12 (12AL)	
<i>L. alpina</i>	2n=36(12AL+24BL)	Agmatoploidy+ Polyploidy
<i>L. calabra</i>	2n=24 (24BL)	Agmatoploidy
<i>L. fallax</i>	2n=24 (24BL)	Agmatoploidy
<i>L. taurica</i>	2n=12 (12AL)	
<i>L. divulgata</i>	2n=24 (24BL), 13 (12AL+1B)	Agmatoploidy
<i>L. campestris</i> s.l.	2n=12 (12AL), 13 (12AL+1B)	
<i>L. sudetica</i>	2n=48 (48CL)	Agmatoploidy
<i>L. congesta</i>	2n=36 (36AL), 48 (48AL)	Polyploidy
<i>L. multiflora</i>	2n=24 (24AL), 36 (36AL)	Polyploidy
<i>L. multiflora</i> subsp. <i>monticola</i>	2n=24 (24BL)	Agmatoploidy
<i>L. mannii</i> subsp. <i>mannii</i>	2n=42	
<i>L. mannii</i> subsp. <i>gracilis</i>	2n=24	
<i>L. abyssinica</i>	2n=24	
<i>L. stenophylla</i>	2n=24 (24BL)	Agmatoploidy
<i>L. capitata</i>	2n=12	
<i>L. oligantha</i>	2n=36	
<i>L. lutescens</i>	2n=12 (12AL)	
<i>L. nipponica</i>	2n=12 (12AL)	
<i>L. leptophylla</i>	2n=12	
<i>L. crinita</i> s.l.	2n=12 (12AL)	

Table 1 contd.

Table 1 contd.

<i>L. crenulata</i>	2n=12 (12AL)	
<i>L. rufa</i> s.l.	2n=12 (12AL)	
<i>L. pumila</i>	2n=12 (12AL)	
<i>L. colensoi</i>	2n=12 (12AL)	
<i>L. decipiens</i>	2n=12	
<i>L. picta</i> s.l.	2n=12 (12AL)	
<i>L. banksiana</i> s.l.	2n=12 (12AL)	
<i>L. meridionalis</i>	2n=12 (12AL)	
<i>L. flaccida</i>	2n=12 (12AL)	
<i>L. densiflora</i>	2n=12 (12AL)	
<i>L. australasica</i> s.l.	2n=12 (12AL)	
<i>L. novae-cambriae</i>	2n=12 (12AL)	
<i>L. modesta</i>	2n=12 (12AL)	
<i>L. acutifolia</i>	2n=12	
<i>L. groenlandica</i>	2n=24 (24AL)	Polyploidy
<i>L. orestera</i>	2n=20 (4AL+16BL), 22 (2AL+20BL)	Agmatoploidy+ Symploidy
<i>L. subsessilis</i>	2n=12	
<i>L. comosa</i> var. <i>comosa</i>	2n=24	
<i>L. comosa</i> var. <i>laxa</i>	2n=12 (12AL)	
<i>L. echinata</i>	2n=12 (12AL)	
<i>L. bulbosa</i>	2n=12 (12AL)	
<i>L. hawaiiensis</i> var. <i>glabrata</i>	2n=28	
<b>Subg. Pterodes</b>		
<i>L. johnstonii</i>	2n=42 (6BL+36CL)	Agmato-dysploidy
<i>L. forsteri</i> subsp. <i>forsteri</i>	2n=24 (24BL)	Agmatoploidy
<i>L. luzulina</i>	2n=24 (24BL)	Agmatoploidy
<i>L. pilosa</i>	2n=62, 66 (66CL), ca.70, 72	Agmato-dysploidy
<i>L. acuminata</i>	2n=48 48CL	Agmatoploidy
<i>L. plumosa</i> subsp. <i>plumosa</i>	2n=46	
<i>L. jimboi</i>	2n=24	
<i>L. rufescens</i> var. <i>rufescens</i>	2n=24, 26, 48, 52 (52CL)	Agmato-dysploidy

Data from Species Plantarum [93] and from the site <http://www.unine.ch/caryo/luzula>

\*Taxa having known chromosome numbers are exclusively indicated.

and *Atlantica* sections. Taxa with a diploid valence can be observed in the other sections, as well as taxa with a variable chromosome number, resulting from the processes of polyploidy and agmatoploidy in the *Luzula* section, and agmatoploidy in the *Diprophyllatae*, *Alpinae* and *Thyrsochlamydeae* sections as well as for the sub-genus *Pterodes*. On the other hand, the two processes of polyploidy and agmatoploidy are not exclusive to the same species, as in the case of *L. alpina* Hoppe with  $2n=36$  (12AL + 24BL), in which the chromosome number results from the combination of the two processes. The monospecific sub-genus *Marlenia* is an exception: *L. elegans*  $2n=6$  (6A<sub>0</sub>L), which presents an original karyotype formed by symploidy (descending agmatoploidy [32]), as does the taxa *L. orestera* (sect. *Luzula*) or *L. parviflora* (Ehrh.) Desv. subspecies *parviflora* (sect. *Diprophyllatae*). With regard to the *Nodulosae* section, at the time of writing no chromosome number has been associated with its species *L. nodulosa* [Bory and Chaub.] E. Meyer.

The phenomenon of agmatoploidy is always associated with the genus *Luzula*, and yet in reality it is not so frequent in the genus, as the bibliographical data shows (Table 1). However, there is a collective species, *L. spicata*, in which agmatoploidy is very common. It presents a wide spectrum of numbers, all originating from the diploid valence ( $2n=12$ , 12AL) by ascending agmatoploidy which has led to improved knowledge of the phenomenon of agmatoploidy.

## AGMATOPOIDY

The work done on duplicated *L. spicata* (García-Herran, in progress) reveals that different chromosome numbers are associated with this complex species, with each demonstrating a very specific distribution (Table 2). The diploid valence  $2n=12$  (12AL) occupies a very disjointed area in Eurasia: southern Jura, several regions of the Alps, Italian Peninsula, Tatry in Slovakia, Chinese Daban Shan, Altai and Tien Shan Mountains. On the other hand, the number  $2n=12$  found in the Upper Atlas Mountains published by Quézel [82], is a particular case since it was not confirmed by Favarger, Galland and Küpfer [25]. Populations offering  $2n=24$  (24BL) are the most widespread, occupying vast regions. They thus extend beyond the Eurasian domain, reaching Greenland and North America. In Eurasia, they occupy the northern territories, Iceland and Scandinavia, all the domain in central and southwestern Europe, Greece, and also North Africa, passing through Corsica. On the other

Table 2. Chromosome numbers of *L. spicata* s.l.

<i>2n</i>	<i>Karyotype</i>	<i>Origin</i>	<i>Author</i>
<b>2n=12</b>	<b>12AL</b>	Jura Mountains	García-Herran [30]
		Upper Alps	García-Herran [31]
		Central Alps	Favarger [24]
			García-Herran [31]
		Eastern Alps (Carinzia)	Nordenskiöld [74]
		Eastern Alps (Slovenia)	Druskovic [19]
		Abruzzi	Favarger (unpublished)
			García-Herran (unpublished)
		South Italy	García-Herran (unpublished)
		Tatry	Michalska [71]
			Murín & Paclova [72]
		Daban Shan (China)	García-Herran [33]
		Altai (China)	García-Herran [33]
		Tien Shan (China)	García-Herran [33]
<i>Agmatoploidy</i>			
<b>2n=24</b>	<b>24BL</b>	Greenland	Böcher [8]
		North America	Nordenskiöld [74]
		Iceland	Löve & Löve [55, 56]
		Sweden	Nordenskiöld [74]
		Central Europe	Nordenskiöld [74]
			Favarger [24]
			García-Herran [30]
		Northern Apennines	García-Herran (unpublished)
		Massif Central	Chassagne [14]
			García-Herran [31]
		Iberian Peninsula	Küpfer [52]
			García-Herran [33]
		Greece	Strid & Anderson [96]
		North Africa	Favarger Galland & Küpfer [25]
Corsica	García-Herran [30]		

Table 2 contd.



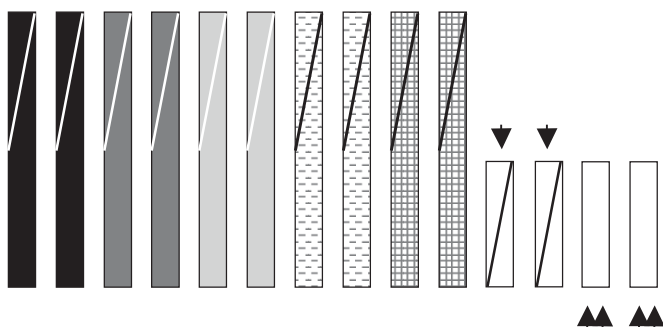
Table 2 contd.

Agmato-dysploidy			
2n=14	10AL+4BL	Eastern Alps (Styria)	Nordenskiöld [74]
		Balkans	García-Herran [30]
2n=16	8AL+8BL	Minor Caucasus	García-Herran [33]
2n=18	6AL+12BL	Great Caucasus	Sokolovskaja & Strelkova [91]
		Upper Alps	García-Herran [33]

hand, intermediate numbers are more isolated and less numerous: 2n=14 (10AL+4BL) in the Styrian Alps and in the Balkans, 2n=16 (8AL+8BL) in the Minor Caucasus, and 2n=18 (6AL+12BL) in the Upper Alps and in the central Great Caucasus.

Until proven otherwise, it can be affirmed that the increase in the chromosome number in *L. spicata* is always attributable to a mechanism of chromosome fragmentation (agmatoploidy). While in certain works the phenomenon is not mentioned, each time authors look into the phenomenon, or when they provide precise comparative images of metaphases, the variation in the chromosome number is confirmed as having its origin in agmatoploidy.

The agmatoploidy is most frequently total (total agmatoploidy [57]) and concerns the two genomes. Consequently, all the chromosomes of a zygotic complement belong to the same size class: in *L. spicata* 2n=24BL derives from 2n=12AL by ascending agmatoploidy and is defined as *agmato-tetraploid*. However, karyotypes with chromosomes in intermediate number have also been observed, between 12 and 24, and including two different sizes. Firstly, the intermediate numbers could be explained by the fragmentation, which affects only one part of the chromosomes (*partial agmatoploidy* or *agmato-dysploidy*). As a result, it is better to distinguish the phenomenon of agmato-dysploidy, in which there are structural arrangements, from the phenomenon of *agmato-aneuploidy* with accidental numbers due to the heterozygotic fragmentations, and even from some relevant cases of polysomy (aneuploidy *sensu stricto* or quantitative aneuploidy). In agmato-dysploidy, the chromosomes, in pair number, all possess a homolog of the same length (Fig. 1). The chromosome number in the population will be fixed and stable, which leads to a variation of the base chromosome number.

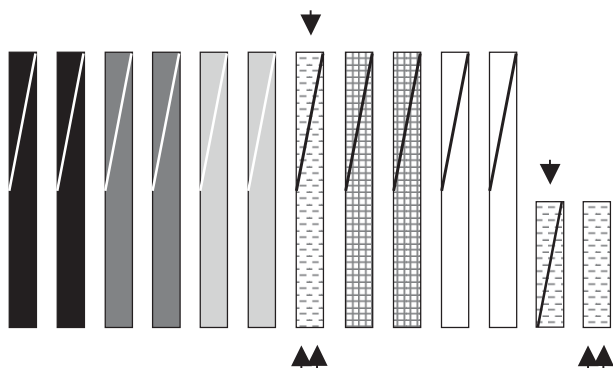


**Fig. 1.** Partial agmatoploidy or agmato-dysploidy, e.g. at  $2n=14$ . The homologies concern chromosomes of the same size. The meiosis will be regular and the cytotype will be the same for all individuals in the population. The genetic equilibrium is respected.

In agmato-aneuploidy, all the chromosomes do not possess a homolog of the same length because the fragmentation affects one of the two chromosomes in a pair of homologues (Fig. 2). Also, the genetic equilibrium in an agmato-aneuploidy is not strongly maintained and the chromosome number will be unstable within a population.

Secondly by the intermediate numbers may well be of hybrid origin. This may be the case with *L. spicata* at several stations analyzed in the Upper Alps, where the individuals at  $2n=18$  (6AL + 12BL) were found in some diploid populations ( $2n=12$ , 12AL). The fact that individuals at  $2n=24$  have not been found is evidence in favour of a new fragmentation. However, it must be added that the populations at  $2n=24$  (24BL) are not very elongated (Alps in Upper Province), making the hypothesis of hybridization feasible. Analyzing the pairings to study the meiosis could in this case concretely define the nature of the intermediate numbers.

The analysis of *L. spicata* provides the foundation for the formulation of a hypothesis regarding the age of the process of agmatoploidy. *L. spicata* is an Alpine species, which is found only locally in the sub-Alpine stage, and rarely in the upper mountain stage. In Mediterranean mountains, it is integrated in formations whose floral composition includes a high proportion of species of central European origin. The establishment of this contingent has been subject to differing evaluations. Quézel [81] states “les grandes glaciations ont apporté à Sierra Nevada (...) un contingent assez important de types orophiles eurasiatiques”. The



**Fig. 2.** Agmato-aneuploidy, e.g. at  $2n=13$ . The homologies concern chromosomes of different sizes. The cytotype will be isolated or more or less in a minority in the population.

migratory movement could even have been prolonged as far as in the Atlas Mountains [83]. In view of the criteria adopted, there is no doubt that Quézel includes *L. spicata* among the glacial immigrants. On the other hand, for the Atlas domain, Galland [29] takes up the conclusions of Contandriopoulos and Gamisans [15], according to whom “les espèces arctico-alpines de Corse sont arrivées dans l’île dès le Tertiaire”. Only the latter point of view can be supported not only because of the paleogeographic but also the biogeographical and karyological facts. Thus, the *L. spicata* complex is represented by the agmato-tetraploidy valence in Corsica, in the Sierra Nevada, and in the Atlas Mountains. Furthermore, in boreal-arctic areas of both the Eurasiatic continent and in North America, *L. spicata* was everywhere subject to karyological control at  $2n=24$  (24BL); and yet, Kulczynski [51] places *L. spicata* precisely in the contingent of the species occupying a large area of America, of southern Europe (Corsica, Sierra Nevada) as well as North Africa since the Tertiary. Evidently, a polytypic origin of the agmatoploidy can be postulated. However, it should be pointed out that in all the aforementioned domains the original populations at  $2n=12$  may have disappeared since it was deduced that the agmato-tetraploid numbers  $2n=24$  (24BL) derive from fragmentation of  $2n=12$  (12AL).  $2n=24$  (24BL) is equally found in *L. abyssinica* Parl. of the central-east African mountains. Even though the affinities between *L. abyssinica* and *L. spicata* s.l. are not discussed by Hedberg [42], they seem to be close. A common origin for the agmato-tetraploids of *L. spicata* and *L. abyssinica* from the same stock at  $2n=24$  cannot be excluded. This hypothesis

would still support the idea of an ancient initial phase of agmatoploidy prior to the extension of the complex *L. spicata* to North America and Africa. The source of agmatoploidy can hardly be determined as there are many uncertainties.

## Agmatoploidy and Polyploidy

In spite of the fundamentally different character of agmatoploidy and polyploidy, on a more or less large scale their biogeographical patterns of distribution do not seem to be very different. In *L. spicata*, all the northern populations linked with the much glaciated regions in both America and Eurasia during the later glaciations possess  $2n=24$  (24BL). It is, therefore, probable that *L. spicata* spread into its northern area from southern regions where diploid populations at  $2n=12$  (12AL) and agmatoploid populations at  $2n=24$  (24BL) coexisted. *L. spicata* represents a complex orophilous Arctic-Alpine agmatoploid from the northern hemisphere [51]. In the same biogeographical domain, the numerous authors inspired by the classic research of Hagerup and Tischler [23, 26, 27, 52, 54, 68, 92,] have presented evidence of different polyploid complexes, being particularly well studied in the Alps and Pyrenees. The principal steps in the history of the orophilous flora are outlined, considering essentially the Quaternary glaciations. In fact, in several complexes of Alpine flora, the diploid race or races occupy the "refuge" territories, only partially affected or totally unaffected by the ice, while the polyploid race or races were able to easily colonize the territories vacated by the glaciers. With regard to polyploid taxon, there is complete lack of knowledge about factors favouring the postglacial extension of *L. spicata* populations at  $2n=24$  (24BL) compared to those at  $2n=12$  (12AL). However, the populations at  $2n=12$  (12AL) in the Jura, Alps, Tatry, and Altai Mountains in any case testify to the existence of potential sources of northward immigration from the lowest valence in *L. spicata*.

In contrast to the polyploidy, it cannot be excluded that agmatoploidy does not confer a selective advantage. The inverse situation is also just as plausible. The fragmentation of the chromosomes does not affect the number of genes (or it is only partially affected). The expression of characters depending on genes with cumulative effect without dominance is, therefore, not modified. If it is difficult to find a selective advantage for agmatoploidy, it is also difficult to find a negative

value. The phenomenon could be totally neutral, in which case, from the orographic and ecological points of view, the colonization of regions left free of ice during the glacial retreat would have initially been achieved by the nearest or the best placed peripheral populations, whatever their chromosome number might be. Perhaps the complexity of the distribution of *L. spicata* populations at  $2n=12$  (12AL) and  $2n=24$  (24BL) in central Europe can be attributed to such a process.

## SYMPLOIDY

All attempts to demonstrate that polyploidy was reversible and that polyploidization played an important role in the evolutionary processes have been very frequently denied. The major reason is the fact that during the course of their evolution, the polyploids suffered mechanisms of genome reorganization resulting in the initial homologies progressively disappearing. In contrast, in the genus *Luzula* symploidy or chromosome fusion (descending agmatoploidy) seems to intervene as an evolutionary mechanism, because it seems that, due to the presence of chromosomes with diffuse centromere, there is no genetic material loss.

*L. elegans* is the only species in the genus which presents the type of annual biology and the lowest chromosome number  $2n=6$ . Its karyological analysis has shown that its chromosomes are of a great size, being more than three times larger than that of diploid taxa. All this leads to the belief that the *L. elegans* chromosomes originate from chromosome fusion. The karyotype  $2n=6$  (6A<sub>0</sub>L) also derives from fusion of all the chromosomes of an endowment at  $2n=12$  (12AL) (total symploidy). Other *Luzulas* exist in which the chromosomal fusion would have been able to intervene: *L. oresteria*. Two chromosome numbers are associated with this species of western North America:  $2n=20$  (16BL+4AL) and  $2n=22$  (20BL+2AL) [74]. The presence of type AL chromosomes could also be explained just as easily by chromosomal fusion as by chromosomal fission. According to Luceño and Guerra [61], the most plausible hypothesis is the one in which these numbers derive from a karyotype  $2n=24$  (24BL) by one or two fusions (partial symploidy): 2BL → 1AL. In the same way it could explain the karyotype of *L. parviflora* var. *parviflora*  $2n=22$  (2AL+20BL) [40]. In order to clarify the origin of this type of karyotype, it would be helpful to understand the mechanisms which “control” the phenomena of chromosomal fusion and fission.

Several authors agree to the great importance of chromosomal fusion in the genus *Carex*. In the opinion of Hartvig [41] *Carex divulsa* Stokes subspecies *leersii* (Kmeuker) W. Koch  $2n=57$  derives from  $2n=58$  by fusion of two chromosomes. According to Luceño [59] the same applies to *C. caryophyllea* Latourr.  $2n=67$  as it derives from  $2n=68$ . Fusion and fission can be present simultaneously as in *C. laevigata* [58], showing that in addition, both can be considered to be evolutionary phenomena. According to Sheikh *et al.* [90] on *D. dichrosepala* Turz, it seems that this could be the case in *Drosera* L. (Droseraceae). However, in nature the phenomenon of chromosomal fusion is not very widespread. Up to the time of writing, it has not been found in other vegetal organisms, and it remains very rare in the animal kingdom [100]. In the light of available data in the literature, chromosomal fusion has only been recognized in certain orders of arthropods: Scorpions [80] and Lepidoptera [98].

## ORIGIN OF THE CHROMOSOME VARIABILITY IN THE GENUS *LUZULA*

It is principally due to the presence of holocentric chromosomes that chromosomal variability can be observed in the genus *Luzula*. This chromosomal variability results from very specific phenomenon such as agmatoploidy, symploidy or polyploidy. This variability implies chromosomal evolution within the genus which might be explained from the diploid valence and primitive valence  $2n=12$  (12AL), possessing the base chromosome number  $x=6$  (6AL). Furthermore, Fig. 3 attempts to show the polarity in the evolution of the chromosome number in the genus *Luzula* DC.

The existence of *L. elegans* at  $2n=6$  (6A<sub>0</sub>L) could support the belief that the chromosomal fragmentation is effected from  $2n=6 \rightarrow 12 \rightarrow 24 \rightarrow \dots$  and also considering  $x=3$  as being the primitive base number. Some data would support this hypothesis. On the one hand, *L. elegans* belongs to a larger centre of biodiversity and to ancient species conservation centre, the Canary Islands and Madeira. Besides, the inflorescence structure in loose anthela with unifloral glomerules, represents a primitive type if one follows the evolutionary scheme proposed by Balslev [2]. On the other hand, *L. elegans* is an annual species introduced into Portugal where it behaves like a “rudérale” species. Nevertheless, these characteristics are generally found in the derived taxa and are rarer in the pale-endemics. In addition, the chromosome number  $2n=6$  is only present in one species. Even though the karyological inventory of *Luzulas*

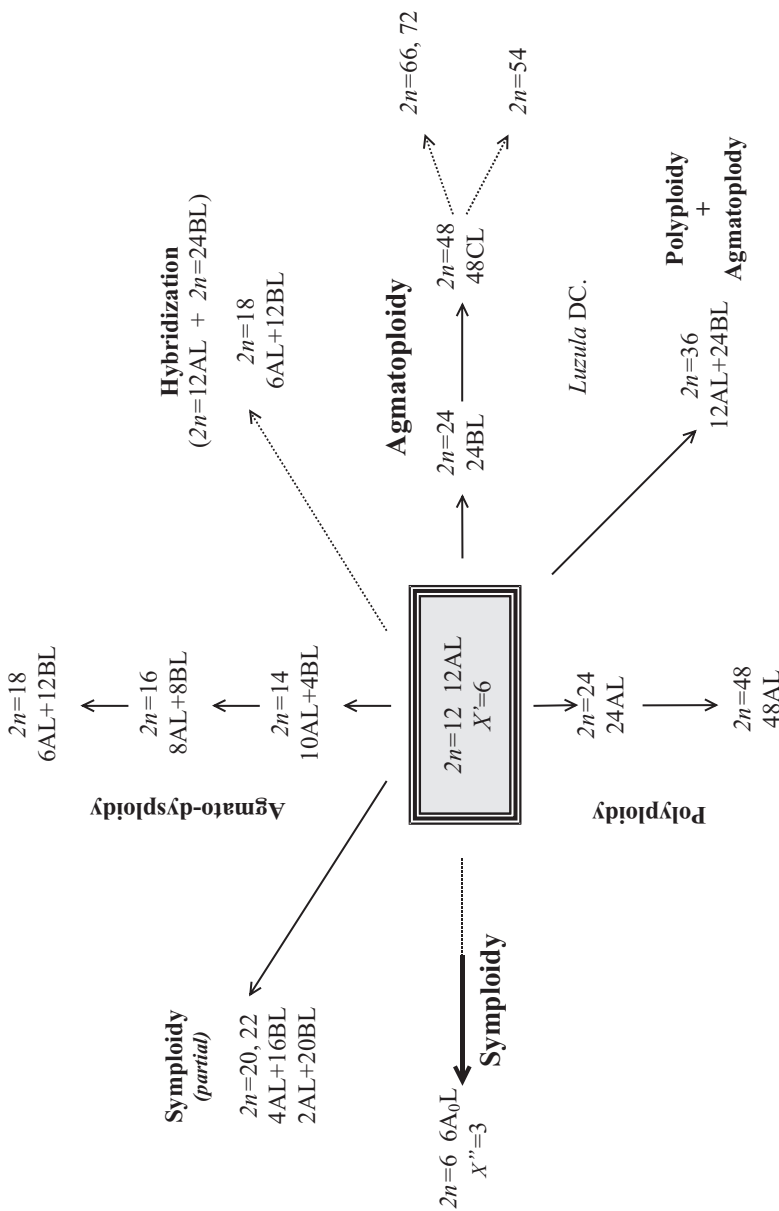


Fig. 3. Origin of the chromosome variability in the genus efface: *Luzula* DC.

has not yet been completed at the time of writing, the data is sufficient to make discovery of other species at  $n=3$  unlikely. If need be, it is still necessary to confirm that the same base number  $x=3$  in the two different species does have symplesiomorphy rather than homoplasy. In angiosperms, to have a base chromosome number lower than  $x=5$  [95, 84] or higher than 11 or 13 [94] is not usual. The fact that chromosome numbers between  $n=3$  and  $n=6$  have not been observed supports the hypothesis of the authors according to which the somatic number 6 would be derived from 12, without doubt from chromosomal fusion  $x=6$  (6AL)  $\rightarrow x=3$  (3A<sub>0</sub>L) or from symploidy. Therefore, the base number  $x'=3$  would have been derived.

Furthermore, it no longer seems particularly conjectural to state that  $x=6$  represents the primitive base number in the genus *Luzula* in view of the widespread distribution, on all continents, of the numbers  $2n=12$  (12AL) which are considered to be the diploid valence. The fact that no species at  $2n=12$  (12AL) in the sub-genus *Pterodes* have been found would validate this hypothesis. All the species are agmatoploid and the number  $2n=24$  (24BL) with  $x'=12$  (12BL) (*L. forsteri* (Sm.) DC. or *L. luzulina* (Vill.) Racib.) appears to be the most ancient of the series. Some species such as *L. pilosa* (L.) Willd.  $2n=62, 66$  (66CL), ca. 70, 72 have undergone several phases of fragmentation. In the opinion of the authors, these data are more a proof of the age of the process of fragmentation rather than the demonstration of the plesiomorphic character of  $2n=24$ . The sub-genus *Pterodes* would be some kind of a paleo-agmatoploid. As indicated above, other examples in the genus lead to the consideration that the agmatoploidy must have intervened very early: the agmato-tetraploid populations of *L. spicata* are disseminated in an almost continuous manner from Morocco to Scandinavia, and from California to the central Alps. Or yet again, the presence of *L. racemosa* Desv.  $2n=24$  (24BL) in America and of *L. abyssinica*  $2n=24$  (24BL) in the mountains of central-eastern Africa are agmato-tetraploid taxa both presenting incontestable affinities with *L. spicata*. The presence of agmato-tetraploids in the Atlas Mountains, in the Sierra Nevada, in Corsica and in North America favors a pre-glacial event inasmuch as the agmatoploidy in the complex *L. spicata* may be monophyletic. In the present state of knowledge, there are still no indications suggesting the contrary.

As a consequence of the diffuse character of the centromere, the rupture of a chromosome does not carry the loss of chromosomal



fragments, or its corollary, the loss of information. Therefore, following what happens in the genus *Carex* [22], the variability of the chromosome number could have been particularly important in the genus *Luzula*. However, karyological analysis demonstrates that the genus is relatively stable, conserving the diploid valence  $2n=12$  (12AL) in a good number of species. This leads to the belief that the *Luzulas* are particularly sensitive to the genetic equilibrium, either on the gametophytic level or on the sporophytic level. A strong selective constraint would favour the structural homozygotes and would eliminate a certain number of genotypes presenting fragmentations in their initial phase, heterozygote. The results presented in this paper tend to support such a hypothesis, the intermediate numbers being less frequent.

The polyploidy seems to be limited to the sub-genus *Luzula*, in which it can present parallel, or combined with the agmatoploidy, as is the case in the *Luzula* section: *L. sudetica* ( $2n=48$  (48CL), [75]), *L. alpina* ( $2n=36$  (12AL+24BL), [49]). The species belonging to this section are the only ones in which the taxonomic treatment is strongly modelled on the karyological data. *L. campestris* is always diploid, whereas polyploid taxa are found in the *L. multiflora* complex. There is an exception in *L. multiflora* subspecies *monticola*  $2n=24$  (24BL), agmato-tetraploid, described by Kirschner [50], who considers them to be the only known European diploid belonging to the *L. multiflora* complex.

Otherwise, agmato-aneuploidy seems to be non-existent in the *Luzulas*, or at least very rare. The supernumerary chromosomes have always been interpreted as B chromosomes [30, 32] from the fact that they presented a different size from that of the AL chromosomes and that they were heterochromatic. The situation seems to be fundamentally different in the genus *Carex* in which, among others, Luceño and Castroviejo [58] and Luceño [60] highlighted aneuploid series (that the authors call agmato-aneuploids) in several species. In particular, the work of the two authors shows that variation was not anarchic: *C. laevigata* presents an agmato-aneuploid gradient with a progressive increase in the chromosome number in the Iberian Peninsula, from the Basque country to the south of Andalusia. Majority of the populations present several different chromosome numbers, the phenomena of fission, incidentally of fusion, still being in a phase, not fully stabilized. Although the genus *Carex* has many similarities with the genus *Luzula*, other differences must be mentioned. Whereas the total agmatoploidy in the genus *Luzula* is very frequent, in *Carex* the variation in the chromosome number can

result from two processes: on the one hand, by addition or by subtraction of one or more chromosomes by chromosome fission or fusion; and on the other hand, by polyploidy [57]. Another distinct characteristic corresponds to the size of the chromosomes. The chromosomal fissions in the *Carex* are most often asymmetric, generating fragments of a generally unequal size. This can be deduced from the explicative scheme for the fragmentation of bivalents in the genus *Carex* [60]. On the other hand, the fragmentation in the *Luzulas* seems to be median. The karyotypes of the agmatoploid are, therefore, relatively symmetrical (García-Herran, in progress), only certain sizes of chromosomes can consequently be observed and associated with the nomenclature of Nordeskiöld [74]. Consequently, the chromosomal variability in the genus *Carex* due to chromosomal fission or fusion can be defined as being agmatoploid, so as to distinguish it from the agmatoploidy (total or partial) observed in *L. spicata*.

If the intermediate numbers in *L. spicata* are closely analyzed, the agmato-dysploidy could have played an important role in affecting the *Luzula* karyotype. In fact, the widespread distribution of number  $2n=14$  (10AL+4BL) in the Balkans supports the belief that the phenomenon may have been monophyletic, old and stabilized, with a variation in the base number. In addition, this number forms part of a progressive gradient of agmatoploid cytotypes towards the east:  $2n=12$  (12AL) (Hohe Tauern, Carinzian Alps),  $2n=14$  (10AL+4BL) (Styrian Alps and Balkans),  $2n=16$  (8AL+8BL) (Minor Caucasus),  $2n=18$  (6AL+12BL) (central Great Caucasus). If this series of numbers is not a fortuitous occurrence, it could result from a partial agmatoploidy, progressive but stabilized. To this can be added the opinions of Malheiros-Gardé and Gardé [67] and Noronha-Wagner and Castro [77], who in their studies of the development of meiosis, explain the progressive character of the agmatoploidy by starting with the presence of intermediate numbers. On the other hand, it is also possible to state that these numbers result from a more recent agmatoploidy, stopping in a still inchoative phase, before perhaps leading to a new taxon at  $2n=24$  (24BL)? Such an interpretation postulates particular affinities between populations of these geographical areas. However, numerical analysis of the *L. spicata* complex in its Eurasiatic area of distribution (García-Herran, in progress) shows no morphological similarity between the individuals of different regions from the eastern Alps to the Caucasus. It

seems that the phenomenon of chromosome fragmentation in the *Luzulas* is significantly different from that presented by *C. laevigata*. Perhaps *Luzula* and *Carex* are found in another phase of their evolution, inchoative for *Carex* and completed in *Luzula*.

Concerning polarity, some facts, however, also speak in favour of the evolution in the genus *Luzula* by chromosomal fragmentation  $2n=12AL \rightarrow 2n=24BL$  rather than by fusion  $2n=24BL \rightarrow 2n=12AL$ . Several species (*L. sylvatica*, *L. lutea*, etc.) present  $2n=12$  (12AL) as a unique chromosome number. Moreover, most of the agmato-tetraploid taxa belong to complexes in which the zygotic number  $2n=12$  still exists. An exception is the sub-genus *Pterodes* and the *L. multiflora* complex. In spite of this, it seems the evolution follows the direction of an increase in the chromosome number by fragmentation from species at  $2n=12$  (12AL) to species at  $2n=24$  (24BL) (ascending agmatoploidy). The inverse polarity, due to symploidy (descending agmatoploidy), is much less frequent. Until now this has been associated with *L. elegans* ( $2n=6 \leftarrow 2n=12$ ) and perhaps with *L. oresteria* and *L. parviflora*. If the cases above are abstracted, the base chromosome number recognized by Nordenskiöld [73] can be agreed with, corresponding to the number of link groups, tending to increase in the *Luzulas*, both by agmato-dysploidy ( $x=6 \rightarrow 7 \rightarrow 8 \rightarrow ? 9$ ) and by agmatoploidy ( $x=6AL \rightarrow x=12BL$ ). It is worth noting that in a different evolutionary stage, the greater polarity (increasing), of the gametic number gradient is essentially the same as that observed in the genus *Carex*. Is the phenomenon fortuitous? Would such pressure of selection interact on the genotype to favour the zygotic numbers above  $2n=12$ ? Questions which for the moment remain open.

## Acknowledgement

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# **PLANT GENOME: BIODIVERSITY AND EVOLUTION**

Volume 1, Part D

**Phanerogams (Gymnosperm) and  
(Angiosperm-Monocotyledons)**

# Phylogenetic Relationships and Systematics in Genus *Bromus* (Poaceae)

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## ABSTRACT

*Bromus* is a taxonomically complex genus with about 130 species of annual and perennial, diploid and polyploid brome grasses of wide geographic distribution. The morphological diversity in the genus *Bromus* and great variation within its species are well-known and have caused substantial difficulties in taxonomic delimitations for various species. Recently many authors have studied genetic relationships and variation of many of the species in the genus *Bromus*, but despite this information, phylogenetic relationships among the subdivisions of *Bromus* are still far from clear. The results obtained from different molecular markers (allozymes, cp DNA, rDNA) highlight the problematic issues for future investigation.

**Key Words:** *Bromus*, taxonomy, phylogenetic relationships

**Abbreviations:** AFLP = Amplified Fragment Length Polymorphism, cpDNA = chloroplast DNA, ITS = Internal Transcribed Spacer, MLAL = Multilocus Allozyme Lineage, MLIL = Multilocus Isozyme Lineage, nrDNA = nuclear DNA, RAPD = Random Amplification of Polymorphic DNA, rDNA = ribosomal DNA, SSR = Simple Sequence Repeats or microsatellites, UPGMA = Unweighted Pair Group Method with Arithmetic Mean.

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INTRODUCTION

The grass genus *Bromus* L. belongs to the subfamily *Pooideae* A. Br. tribe *Bromeae* Dum. It contains herbaceous self- and cross-pollinating species of different ploidy levels with the basic chromosome number  $x = 7$ , and has long been known for interspecific hybridization, variability, and taxonomic complexity [100]. Polyploid species with chromosome numbers as multiples of 14 occur in all of sections of the genus [15]. The genus *Bromus* probably originated in western Eurasia [83, 100] and is now extremely widespread throughout Eurasia, extending to North and South America, Africa and Australia. *Bromus* is a taxonomically complex genus with about 130 species of annual and perennial, diploid and polyploid brome grasses of wide geographic distribution. Phylogenetically, it has been shown [29, 93] to be basally sister to the tribe *Triticeae* Dum., that contains economically important crops – barley, rye and wheat. Brome-grasses may give suitable genes for the improvement of different agronomic properties of the cereal crops, e.g. disease resistance and tolerance to various stressful conditions [64].

The genus has been divided in various ways either into seven sections [84], seven subgenera [100], or even seven different genera [102] as summarized in Table 1. The number and rank of the divisions depend on the characters used as a basis of their delimitation (serological, cytological and morphological, respectively), and phylogenetic relationships among them are still inconsistent. Sales [74] doubted the reality of the section *Genea* as an independent taxonomic unit. She noted that there is a continuous range of variation between sections *Bromus* and section *Genea* via the *B. pectinatus* complex of

Table 1. Genus *Bromus* taxonomic delimitation by different authors

Tzvelev (1976)	Smith (1972, 1985)	Stebbins (1981)
- 7 genera	- 7 sections	- 7 subgenera
Anisantha	Genea	Stenobromus
Bromus	Bromus	Bromus
Bromopsis	Pnigma	Festucaria
Ceratochloa	Ceratochloa	Ceratochloa
–	Neobromus	Neobromus
Boissiera	Boissiera	Boissiera
Nevskiella	Nevskiella	Nevskiella
Littledalea		

section *Bromus*. This complex was suggested to link the *Genea* species with the section *Bromus* through the diploid *B. japonicus* [74, 80, 84].

Some aspects of genetic relationships in genus *Bromus* have been investigated using molecular methods with cpDNA and rDNA markers [4, 65, 68]. Pillay and Hilu [68], on the basis of the cpDNA data, found that subgenera *Stenobromus* and *Bromus* are not distinct entities and probably originated from similar ancestors related to the subgenus *Festucaria*. They examined cpDNA restriction site variation in 32 species, representing five subgenera of *Bromus*. The analyses indicated two major clades within the genus. One clade includes subgenus *Neobromus* and subgenus *Ceratochloa*. The other is composed of subgenera *Festucaria*, *Stenobromus* and *Bromus*. Species of subgenus *Festucaria* appeared in three separate lineages with very little resolution of relationships between them. Pillay [65] also concluded that it was unlikely that the subgenera vs. sections of *Bromus* had independent origins. However, Ainouche and Bayer [4] showed sections *Pnigma*, *Genea* and *Bromus* in separate monophyletic clades on the rDNA ITS sequence cladogram. Thus, despite this molecular information, complete detailed clarification is still required regarding phylogenetic relationships between the subdivisions of *Bromus*.

In addition to inconsistent intrageneric classification, there are a lot of problems about the species delimitation within the sections, due to a great and frequently continuous morphological variability and less known genetic relationships between them. Among the diploids, some species are easily recognized by unique morphological characters, like *B. pumilio* and *B. danthoniae*, but other species are hard to identify because their diagnostic characters are often overlapping and difficult to define [84]. The morphological diversity in the genus *Bromus* and great variation within its species are well-known and have caused substantial difficulties in taxonomic delimitations for various species. The high degree of phenotypic plasticity in morphology depending on the environmental conditions, complicate the use of morphometric characters for species identification.

## Section *Genea*

Section *Genea* Dum [86], synonyms: subgenera *Stenobromus*, genera *Anisantha*, of the genus *Bromus* L. comprises weedy, annual brome grasses widely distributed in Mediterranean countries, Southwest Asia,

extending to northern Europe. The centres of diversity of section *Genea* are in the Mediterranean region.

Section *Genea* has been regarded as an evolutionarily advanced section in the genus *Bromus* that is still in the process of further specialization [76]. Species of the section often display remarkable inter- and intraspecific variation in morphology. The section includes the following species: diploids ( $2n=14$ ) *B. sterilis* L., *B. fasciculatus* Presl and *B. tectorum* L., tetraploids ( $2n=28$ ) *B. rubens* L. and *B. madritensis* L., hexa- and octoploid *Bromus diandrus* Roth ( $2n=42, 56$ ) and *B. rigidus* Roth ( $2n=42, 56$ ) with two intraspecific ploidy cytotypes [18, 19, 34, 36, 63, 86, 100, 102]. Naganowska [51] studied karyotypes of five *Bromus* species of *Genea* section. She revealed significant differences in the chromosome features between diploids *B. fasciculatus*, *B. sterilis* and *B. tectorum* and similarities between tetraploids *B. madritensis* and *B. rubens*. Genome affinity between diploid *B. tectorum* and tetraploids *B. madritensis* and *B. rubens* was also found.

It has been supposed [74, 80] that sections *Genea* and *Bromus* may be less distinct than recognized before. Analysis of flower microstructural variation within the genus *Bromus* showed [39] that sections *Bromus* and *Genea* are evolutionarily close and they distinctly overlap each other in the minimum spanning tree space. Cladistic analysis of isozyme variation data among the *bromus* species nested the two *Genea* diploids *B. tectorum* and *B. sterilis* among the diploids of section *Bromus* [60]. Sales [74] questioned the reality of the section *Genea* as an independent taxonomic unit. Following the previous Scholz's study [80], she also suggested that sections *Genea* and *Bromus* may be linked through *Bromus pectinatus* of the section *Bromus*. Sales [74] supposed that *B. pectinatus* of section *Bromus* is closely related to section *Genea*. The placement of *B. pectinatus* in section *Bromus* is also questionable because the characters of its spikelets (wedge-shaped, broader to the top) clearly match with those of the section *Genea*. Scholz [81] in his study about the *B. pectinatus* complex suggested the hybrid origin of this complex. The morphological characteristics of *B. pectinatus* are something between *B. japonicus* of section *Bromus* and *B. tectorum* of section *Genea*. This species resembles *B. tectorum* in general morphology and may have taken part in the species formation in *Genea*. The descriptions of *B. pectinatus* [69, 74, 81] often mentioned the robustness of this species, which may be due to its tetraploid nature [49, 84]. Kosina [38], on the basis of 14 parameters of the embryo morphology, also confirmed the intersectional status of *B.*

*pectinatus*. Isozyme data confirm the allotetraploid nature of *B. pectinatus* and its intermediate position between section *Genea* and section *Bromus*. Several heterozygotes are consistent with the view that *B. pectinatus* may be the result of the hybridization between *B. japonicus* of section *Bromus* and *B. tectorum* of the section *Genea* (Oja, in preparation).

*Bromus tectorum* L. and *B. sterilis* L. are annual diploids that are predominantly cleistogamous, colonizing weeds. They often share the same habitats and grow intermixed and sympatrically, but are easily distinguishable based on morphology [74, 76, 84]. The two species are clearly differentiated karyotypically [40], indicating their reproductive isolation and specific distinctness. All three diploid species of section *Genea* have diverged from each other at a number of isozyme loci [42, 57]. Genetic diversity among 50 accessions of *Bromus tectorum* and 43 of *B. sterilis* from different sites of their Eurasian ranges has been studied by electrophoretic analysis of ten enzymes encoded by 18 loci by Oja [55]. The two species proved clearly differentiated by alternate allozymes of seven isozymes. Populations of both taxa showed differentiation into eleven (*B. tectorum*) and six (*B. sterilis*) multilocus allozyme lineages (MLALs). The extent of interspecific allozyme divergence estimated by Manhattan distance exceeded more than three times intraspecific differentiation between the multilocus lineages. Only two MLALs in each species have wide geographical distribution from Near East to Europe. Other MLALs were found each for only one or two populations and were region-specific. Most geographically marginal European populations had widespread MLALs. Nuclear microsatellite analysis of *Bromus sterilis* from three English farms [27] revealed that *B. sterilis* exists as numerous separate and genetically different lines, which are maintained by inbreeding with occasional outcrossing.

*Bromus tectorum* was introduced into North America, South America, Australia and New Zealand, where it rapidly developed into a noxious weed. Novak et al. [53] detected only low allozyme variation within *B. tectorum* populations in North America. Bartlett et al. [16] found that the invasion of North America by *B. tectorum* occurred through multiple introductions on both coasts. Eurasian populations of *B. tectorum*, however, exhibited somewhat greater allozyme diversity than those in North America, and most differentiated were populations from Southwest Asia, the presumed ancestral native region of this species [52]. This is consistent with the view that the majority of allozyme diversity in autogamous species occurs between populations and that

within-population diversity is, as a rule, relatively low [28]. Ramakrishnan et al. [71] using SSR and AFLP data, showed the presence of significant genetic variation among *B. tectorum* populations. They described 40 self-pollinating lines from four populations of *B. tectorum* in the US and suggested that the present genetic diversity is the result of selection in original founder populations rather than the result of mutations.

Pillay and Hilu [68], on the basis of the cpDNA restriction site data, suggested that *B. sterilis* and *B. tectorum* are recently derived species that share a common maternal ancestry.

Among diploids, *B. fasciculatus* is sporadically distributed in the Mediterranean region and has a more restricted range, being confined to East Mediterranean and western parts of Southwest Asia. In contrast with other species of section *Genea*, *B. fasciculatus* has quite a uniform morphology. The general habit of *B. fasciculatus* shows resemblance to tetraploid *B. rubens* and to depauperate specimens of tetraploid *B. madritensis*, but the diploid can be easily distinguished from both tetraploids by very narrow, needlelike glumes and lemmas, and by strongly outcurved, often twisted, narrow grains. *Bromus fasciculatus* is most distinct among diploids with nine species-specific allozymes [56]. A characteristic feature of isozyme variability in three diploid bromes is almost total absence of two- or three-banded heterozygous allozyme phenotypes at polymorphic isozymes. All variations were observed as single-banded electrophoretic variants attributed to allozymes, indicating that selfing is characteristic of all diploids examined [56, 60]. This is in accordance with other data on autogamy and self-fertilization of *Genea* bromes [43, 44, 53, 84].

The polyploids of the section *Genea* are mainly or exclusively Mediterranean taxa. *Bromus madritensis* L. sensu lato, including *B. madritensis* L. subsp. *kunkelii* H. Scholz together with *B. haussknechtii* Boiss., *B. flabellatus* Boiss., and *B. rubens* L., form a complex of morphologically similar taxa with small lemmas and erect, more or less contracted panicles at flowering time. These characters separate the *Bromus madritensis* complex from the remaining species in section *Genea*. All members of this complex are annual, predominantly self-fertilizing tetraploid grasses with  $2n=28$  [51, 63, 75]. *Bromus rubens* showed no outcrossing [30, 31].

Species belonging to the complex exhibit continuous morphological variation ranging from typical *B. madritensis* (with longer panicle

branches and looser panicles) to typical *B. rubens* (with brush-like condensed panicles), with *B. flabellatus* Boiss, *B. haussknechtii* and newly described by H. Scholz [80] *B. madritensis* subsp. *kunkelii* showing intermediate characters. *Bromus madritensis* and *B. rubens* have traditionally been recognized as separate species on the basis of the panicle and spikelet characters, whereas *B. haussknechtii* and *B. flabellatus* have mostly been considered only as subspecies of *B. madritensis* [62, 73, 79]. The great morphological diversity within the group has also been explained by phenotypic plasticity depending on the environmental growth conditions [19, 20, 22, 76, 106].

Isozyme electrophoresis was used to study genetic diversity and divergence among the brome grass species of the *B. madritensis* complex, comprising *B. madritensis* s.l. and *B. rubens* [57]. In total, 40 multilocus isozyme lineages (MLILs) were determined for 152 accessions of the complex from different sites throughout their geographic distribution. Two most common lineages were found for *B. madritensis* and one for *B. rubens*. At the isozyme level, a clear division corresponding to the two species was evident by species-specific phenotypes of four isozymes. Cluster analysis based on isozyme phenotypes also supported the recognition of two species in the complex. The UPGMA dendrogram showed that *B. madritensis* MLILs could be divided into two major groups or putative subspecies, but they did not correspond to differentiation by morphological characters. Morphologically defined *B. madritensis* subsp. *kunkelii* was clubbed together with *B. rubens* in the same cluster, supporting the recognition of this subspecies within *B. rubens*, not under *B. madritensis*.

Isozymes have been frequently employed to establish allo- versus autopolyploid nature of plant polyploids, and to identify their putative diploid progenitors [17, 26, 92]. Allopolyploids are characterized and distinguished by fixed enzyme heterozygosities derived from their diploid progenitors and show disomic inheritance [32, 72]. Kahler et al. [32] found that electrophoretic phenotypes of enzymes could help to distinguish the *Genea* species. The two tetraploids, *B. madritensis* and *B. rubens* exhibited different fixed heterozygosities of several heterozygotes, indicating their allopolyploid nature and independent origins [59]. Oja [56] has analyzed and compared allozyme variation of three diploid species with that for polyploid species of section *Genea*. Allozymes characteristic for diploids *B. tectorum* and *B. fasciculatus* are combined in fixed heterozygous phenotypes of tetraploid *B. rubens*, suggesting that tetraploid *B. rubens*



might have been derived from a hybrid of *B. fasciculatus* with *B. tectorum*. Fixed heterozygous phenotypes of tetraploid *B. madritensis* combine one allozyme of *B. fasciculatus* with another of diploid *B. sterilis* at each of the loci studied. *Bromus fasciculatus* thus appears to be a third diploid ancestor for the two tetraploids of section *Genea*, *B. rubens* and *B. madritensis*.

*B. diandrus* and *B. rigidus* are indigenous to the Mediterranean region, *B. rigidus* is restricted more to coastal regions than *B. diandrus*. Kon and Blacklow [37] showed that outcrossing in *B. diandrus* was less than 1%. *Bromus diandrus* and *B. rigidus* have been reported as polyploids with different intraspecific ploidy levels: *B. diandrus* ( $2n = 28, 42, 56$ ) *B. rigidus* ( $2n = 28, 42, 56, 70$ ) [21, 35, 77]. *Bromus diandrus* and *B. rigidus* are morphologically quite variable [23, 74]. Although traditionally *B. diandrus* and *B. rigidus* are usually treated as separate species, some authors do not agree. Ovadiahu-Yavin [63] proposed their recognition as two subspecies of *B. rigidus*. Esnault and Huon [24] supposed to consider them as members of a polyploid complex with hexa- and octoploid cytotypes. Sales [74] had treated these species as two different varieties of *B. diandrus* because of the occurrence of a lot of individuals with intermediate characters and unclear geographical and ecological separation. The existence of intermediate types makes identification difficult, and morphological characters may not alone permit resolution of these species. Isozyme data [59] also showed that *B. diandrus* and *B. rigidus* are genetically closely related and do not deserve the rank of separate species. Almost all brome grass investigators have had complications in distinguishing the taxa solely on the basis of overall morphology in the *B. diandrus-rigidus* polyploid complex. Chromosome numbers, morphological characters and isozymes of seven enzymes were studied by Oja and Laarmann [62] to assess relationships between species of the *Bromus diandrus-rigidus* polyploid complex and *B. sterilis* that is a closely related diploid ( $2n = 14$ ) species [20, 40, 78]. It has been shown to be a putative genome donor for the *B. diandrus-rigidus* polyploid complex by the isozyme evidence [59]. Some authors give two chromosome numbers for *B. sterilis*,  $2n=14$  and  $2n=28$  [20, 48]. *Bromus sterilis*, *B. diandrus* and *B. rigidus* are quite similar in overall morphology, the last two being more robust plants [74, 76, 83, 86]. The four different cytotypes detected,  $2n=14, 28, 42$ , and  $56$ , could be divided into two species: *B. sterilis* ( $2n=14, 28$ ) and *B. diandrus* ( $2n=42, 56$ ) by morphological features and isozymes [62]. The shape of the scar of

rachilla segments in the floret proved to be a suitable character for distinguishing the two species, but not for distinguishing the chromosomal races within species. The tetraploid shared homozygous isozyme phenotypes with diploid *B. sterilis* at all loci except one, suggesting that it is autopolyploid. No diagnostic isozymes could be found to distinguish between hexa- and octoploid cytotypes in the *B. diandrus-rigidus* complex.

Isozyme electrophoresis was also used to study the origin of the *B. diandrus-rigidus* polyploid complex. *Bromus diandrus* and *B. rigidus* revealed identical zymograms, providing new support for their conspecific recognition. Different fixed heterozygosities of several heterozygotes suggest their allopolyploid nature and independent origin from different diploid progenitors [59]. The pattern of isozyme variation evidence against the participation of diploids *B. fasciculatus* and *B. tectorum* and tetraploids *B. rubens* and *B. madritensis* in the hybrid origin of the hexa-octoploid *B. diandrus-rigidus* complex [57]. Of the three diploids studied, only *B. sterilis* fits well for a role of a genome donor for the polyploid *B. diandrus-rigidus* complex, while the diploid progenitors of other genomes yet remain to be established.

## Section *Bromus*

Section *Bromus* of the genus *Bromus* L. [87], synonyms: subgenus *Bromus*, genus *Bromus*, together with section *Genea* is considered to be the most advanced section in the genus that probably arose during the Pleistocene in southwestern Asia and the Mediterranean area [100]. It includes annual or biennial, predominantly self-fertilizing species that are widespread in Eurasia. Many of them have been distributed into the New World, Africa and Australia [73, 83, 100]. Southwest Asia and Eastern Mediterranean area are considered as the centre of diversity for this group [84, 74]. The number of species included in the section varies remarkably (from 30 to 40), depending on the author of the classification and on the accepted synonymy [79, 84, 102]. Polyploidy is common in this section and is restricted to the tetraploid level [100]. The species of the section *Bromus* have the largest genome size in the genus [15]. Most of species are well-known weedy grasses with wide geographic distribution. Several features, including colonizing success, morphological variability, phenotypic plasticity, hybridization, and polyploidy, make this group of considerable evolutionary interest [100]. Section *Bromus*

contains several species complexes with taxonomically problematic species which are closely related and difficult to delimit, e.g. the *B. mollis* s.l. complex [82] or *B. commutatus* and *B. racemosus* [85]. Already Linnaeus was in trouble with species delimitation in section *Bromus*. He changed his concept of some brome species several times [82]. Later, it was one of the reasons for a number of misdescriptions. This section has been regarded as a taxonomically difficult group for a long time and even nominated as “taxonomic nightmare” [88]. All authors realize the difficulty of assessing interspecific relationships and phylogeny in section *Bromus* by using only morphological characters, as is typical for many grasses [33].

Ainouche and Bayer [3] found a weak divergence among the diploid species within the section *Bromus* by the ITS sequences of the nrDNA. Phylogenetic relationships and genetic differentiation between some diploid annual brome species were evaluated by cladistic and phenetic analysis of allozyme diversity performed by Oja and Jaaska [59]. The diploids of the section *Genea* and section *Bromus* were distinguished into separate subclusters on both cladistic and phenetic allozyme trees. Diploids *Bromus pumilio*, *B. danthoniae*, *B. scoparius* and *B. alopecuros*, despite intraspecific allozyme polymorphism of several heterozymes, lacked heterozygous allozyme phenotypes, indicating prevalent self-fertilization. The important consequence of autogamous breeding system in these diploid bromes is their intraspecific differentiation into distinct multilocus isozyme lineages. Two more distinctly differentiated species among diploids of section *Bromus* seem to be *B. pumilio* and *B. danthoniae*, which are placed basally paraphyletic to the other species of the section *Bromus*. Smith [90] also supposed that *B. pumilio* is most closely related to *B. danthoniae* and *B. alopecuros*. The clear allozyme differentiation of *B. danthoniae* is congruent with its divergence by a unique morphological character (this species has three-awned lemmas).

*Bromus arvensis*, *B. japonicus* and *B. squarrosus* are morphologically similar diploids of section *Bromus* with largely overlapping species descriptions and inconsistent taxonomy. In the first description of *B. japonicus*, Thunberg [103] emphasized its close resemblance to *B. arvensis*, whereby having oblong spikelets and divaricated awns differentiates it. Taxonomists have always recognised that *B. japonicus* is a greatly variable taxon with at least two subspecies: typical subsp. *japonicus* Thunb. and subsp. *anatolicus* (Boiss & Heldr.) Penzes. The

descriptions of subsp. *anatolicus* and of *B. squarrosus* are obviously overlapping [86, 89]. Acedo and Llamas [2] in their monograph about genus *Bromus* in the Iberian Peninsula, suggest that *B. japonicus* is nothing more than *B. arvensis* with cleistogamous florets and small anthers and, therefore, with selfing breeding behaviour, respectively. Krechetovich and Vvedensky [41] placed *B. arvensis* in a new series *Macrantherae*, and positioned *B. japonicus*, *B. squarrosus*, *B. anatolicus* Boiss. et Heldr. and *B. briziformis* Fisch. et Mey. in another new series *Squarrosae*. This system was later followed by Scholz [79] and supported by Smith [84], Tzvelev [102] and Stace [97]. Most Floras still recognize three different species *B. japonicus*, *B. squarrosus* and *B. arvensis* and may underestimate the presence of remarkable variability with intermediate morphological forms between them. *Bromus arvensis* has a wide geographic range from Southwest Asia, throughout the Mediterranean area to Northern Europe [86, 89]. *Bromus japonicus* and *B. squarrosus* are also widely distributed throughout the Mediterranean region, but extend to central Asia and have been introduced to America and Australia [89].

Serological study [84] showed that *B. squarrosus* and *B. japonicus* are closely related, whereas *B. arvensis* is distinct from them. The RAPD [5] and ITS data [4] also showed that *B. squarrosus* and *B. japonicus* are sister species but *B. arvensis* is somewhat different. Isozyme results [61] revealed that *B. japonicus* and *B. squarrosus* do not have species-specific isozymes and do not form separate clusters on the dendrograms based on the isozyme data. *Bromus arvensis* has two species-specific allozymes and is distinguishable from *B. japonicus* and *B. squarrosus*.

Estimates of outcrossing rate ( $t=1$ ) in *B. arvensis*, indicating essentially complete allogamy with random mating, whereas *B. japonicus* and *B. squarrosus* ( $t=0.00$ ) are extreme selfers. It is suggested that *B. japonicus* and *B. squarrosus* are autogamous relatives of the outcrosser *B. arvensis*, but not its immediate progenitors [61]. Twenty-four multilocus isozyme lineages (MLILs) were detected among the 42 accessions of *B. japonicus* and *B. squarrosus*, with some MLILs containing accessions of both taxa. No geographic pattern was found among the accessions or MLILs of *B. japonicus* and *B. squarrosus*.

According to classificatory discriminant, canonical discriminant, principal component and cluster analyses of the morphological characters, the accessions of *B. arvensis*, *B. japonicus* and *B. squarrosus* were separated into three moderately distinct groups that corresponded

to the three traditional species. The results showed that qualitative characters were the best for the delimitation of the taxa by statistical analyses.

*Bromus intermedius* Guss. is an annual diploid ( $2n=14$ ) that has mostly Mediterranean distribution: Southern Europe, Northern Africa and Southwest Asia. In the Iberian Peninsula it is found only in the southern part of Spain [2]. The main difference between *B. japonicus* and *B. intermedius*, according to a recent very accurate key to Bromaeae in the Mediterranean climatic zones [96], is the dry lemma texture which is papery, usually with protruding veins in *B. intermedius* and leathery, usually without protruding veins in *B. japonicus*. Inflorescence structure, panicle branch length and spikelet measurement are greatly influenced by environmental conditions during the vegetation period [89, 91] and are of limited value to distinguish the two species. Unfortunately, pubescence can hide protruding veins and frequently in *B. japonicus* leathery lemmas can be quite thin and thus with protruding veins. Spalton emphasized this fact in the notes of his paper [96]. *Bromus japonicus*, *B. squarrosus* and *B. arvensis* form a similar cluster of closely related species [61], and *B. intermedius* also belongs to this complex. Genetic diversity and differentiation among the *B. intermedius* accessions of different geographic origins have been recently studied by me, using isozyme analysis. The mating system was evaluated on the basis of allozyme polymorphism. Outcrossing rate ( $t$ ) in *B. intermedius* was mostly 0, except one population with  $t = 0.16$ , indicating nearly complete autogamy in this species. Given that *B. arvensis* and *B. intermedius* had common allozymes of all isozymes studied, it is suggested that *B. intermedius* may be a direct autogamous derivative of the outcrosser *B. arvensis*. Contrary to expectations, the allozyme diversity in selfing *B. intermedius* was higher than in outcrosser *B. arvensis*, comprising 23 and 16 allozymes, respectively [58].

An artificial group of 12 brome grasses with small spikelets was reviewed by Smith and Sales [91]. They supposed that four small-spikelet bromes (*B. brachystachys*, *B. pseudobrachystachys*, *B. lepidus* and *B. scoparius*) would be the most highly evolved species in the section *Bromus*.

*Bromus racemosus*, *B. commutatus* and *B. secalinus* are related tetraploids ( $2n=28$ ) of the section *Bromus*. Commonly they are recognized as different species, however some authors [2, 7, 45, 50]

treated *B. commutatus* as a subspecies of *B. racemosus*. Smith [85] admitted that they are not identical but overlap ecologically. Lloret [46] regarded *B. commutatus* as a subspecies of *B. secalinus*. Morphologically, *B. commutatus* appears to be in a central position between *B. racemosus* and *B. secalinus*. Spalton [95] supposed that "these three taxa may have originated from an unidentified common diploid ancestor which may no longer be existing". He also analyzed the morphological characters traditionally used in identification of these taxa and proposed a new detailed key for them. Allozyme data [54] show that tetraploids of section *Bromus* exhibited different fixed heterozygosities of several heterozyms, suggesting their allopolyploid nature and independent origins. *Bromus secalinus* and *B. commutatus* displayed homologous variation with shared morphs at several heterozyms, indicating their strong genetic affinity. The isoenzyme results on the close phylogenetic affinity of *B. secalinus* and *B. commutatus* are consistent with the morphological similarity of these species, their serological affinity [84] and hybridization data [105]. At the same time, a marked difference between them was revealed by one isozyme and it supports their recognition as separate species.

Ainouche et al. [6] studied allozymic genetic diversity among the four species belonging to section *Bromus* in the Mediterranean region. *Bromus intermedius*, *B. squarrosus*, *B. lanceolatus* and *B. hordeaceus* displayed substantial genetic similarity. At the same time, the species were clearly differentiated with tetraploid *B. hordeaceus* being more closely related to diploid *B. squarrosus*, and tetraploid *B. lanceolatus* to diploid *B. intermedius*, respectively. They found that self-fertilizing diploids *B. intermedius* and *B. squarrosus* may have substantial levels of allogamy and are genetically less diverse than widespread tetraploids *B. lanceolatus* and *B. hordeaceus*.

The diploid *B. pseudosecalinus* is morphologically very similar to the tetraploid *B. secalinus*. The two species were found to be serologically very different [84], and allozyme results [54] also showed that *B. pseudosecalinus* could not be one of the diploid progenitors for *B. secalinus*.

Smith [84] placed *B. secalinus* and *B. alopecuroides* in separate groups on the basis of morphological and serological differences. On the other hand, Pillay and Hilu [68] suggested close relationships between them on the basis of the cpDNA. Allozyme results [54] suggest that *B. alopecuroides* does not suite as a diploid parent for tetraploid *B. secalinus*. Smith [84]

concluded that *B. danthoniae* is likely to be one of the diploid ancestors of *B. lanceolatus* because of the serological similarity. This is, however, not supported by isozymes. Instead, allozyme data [54] indicate that *B. alopecuroides* could be one of the genome donors for the tetraploid *B. lanceolatus*.

*Bromus hordeaceus* is an allotetraploid predominantly self-pollinated invasive weed with a very wide distribution [100]. Traditionally, four subspecies were recognized in the morphologically considerably varying *B. hordeaceus* complex. Three of them are habitat specific “ecotype-subspecies”: subsp. *ferronii*, subsp. *thomii* and subsp. *molliformis* [87]. *Bromus hordeaceus* sp. *hordeaceus* (syn. *B. mollis* L.) is a type subspecies that grows in very different ecological and geographical conditions. Outcrossing rate in *B. hordeaceus* differed from 1-18% [30, 31] in different geographical regions. Lönn [47] reported that *B. hordeaceus* is a largely self-fertilizing species with restricted gene flow, because only one heterozygote out of 239 electrophoretically screened individuals was found in Öland, Sweden.

Ainouche et al. [3] have shown that the interpopulational differentiation within *B. hordeaceus* is based on geographic, rather than on subspecific taxonomic divergence. Similarly, intraspecific allozyme variation observed in *B. hordeaceus* [54] was independent from the morphological differentiation that recognized three ecotype-subspecies in *B. hordeaceus*. Ainouche et al. [5] studied genetic diversity at enzyme loci and ITS sequences from the nrDNA in 15 Mediterranean and Atlantic populations of *B. hordeaceus* and found no genetic differentiation among the four subspecies. All the populations studied were homozygous, suggesting selfing. The tetraploids *B. hordeaceus* and *B. interruptus* are very close morphologically. In addition to this external resemblance, they showed a 75-80% similarity in seed proteins [84] and shared identical isozyme phenotypes [54]. Thus, isozyme data support the suggestion of Smith [87] that *B. interruptus* may be interpreted as an ecotype-subspecies of *B. hordeaceus*.

Spalton [94] has described a new subspecies *B. hordeaceus* subsp. *longipedicellatus* that resembles *B. commutatus* in general appearance. He found that *B. hordeaceus* subsp. *longipedicellatus* is most closely related to *B. hordeaceus* subsp. *hordeaceus* and could be distinguished from the latter by having some pedicels and branches longer than the spikelets that they bear. Spalton supposed that subsp. *longipedicellatus* may be the result of a



gene transfer from *B. hordeaceus* into *B. racemosus* or *B. arvensis* with *hordeaceus* genes becoming dominant. Before arriving at a definite conclusion about a gene transfer between *B. hordeaceus*, *B. racemosus* or *B. arvensis*, an analysis of the extent to which these species are self-fertilizing or cross pollinated, should be made. Wilson [105] produced fertile interspecific hybrids of *B. racemosus* and *B. commutatus*, which were morphologically intermediate between the parents and proposed to group them indiscriminately as one species. Smith [85] also suggested that *B. commutatus* and *B. racemosus* hybridize in Britain but argued against lumping them. Ainouche et al. [6] reported substantial amount of allogamy for *B. hordeaceus*. Oja et al. [61] estimated outcrossing rate of *B. arvensis* as approximately  $t = 1$ , indicating that *B. arvensis* is a complete outcrosser with random mating. Thus, the high level of gene flow in these taxa is real and Spalton's hypothesis could be true, but needs more evidence, perhaps more effectively from molecular markers.

Acedo and Llamas [1] described two new annual brome grasses from the Iberian Peninsula: *Bromus cabrerensis* and *B. nervosus*, belonging to section *Bromus*. *Bromus cabrerensis* is a tetraploid and closely related to *B. hordeaceus*, from which it clearly differs by large panicles with numerous spikelets. *B. nervosus* has a rather isolated position within section *Bromus* and its chromosome number and other features are not studied yet, as it is only known from two herbarium specimens.

## Section *Pnigma*

Section *Pnigma* [83], synonyms: subgenus *Festucaria* and genus *Bromopsis*, is the largest section in the genus *Bromus*. It contains about 60 perennial, predominantly outcrossing species with two major centres of distribution in Eurasia and North America. Eurasian species of the section *Pnigma* are mainly polyploids (11 species), only 3 diploid species are known there. High level of polyploidy, predominantly hexa- or octoploid, is characteristic for *Pnigma* species in Eurasia [98, 100]. On the other hand, polyploidy is less frequent in the North American species of the section *Pnigma* and is mostly limited at the tetraploid level. The majority of North American species are diploids. Two endemic species from South America are hexaploid (*B. auleticus* and *B. uruguayensis*). Relationships of these species to the other species of the section *Pnigma* are unknown [15]. Pillay and Hilu [67], on the basis of the cpDNA data, confirmed the monophyly of section *Pnigma* that has been proposed by Stebbins



[99] and Armstrong [11]. Cytotaxonomic studies of hybrids between *Pnigma* species by Armstrong [8, 9, 100] revealed that *B. pumpellianus* is an autoallopolyploid with an AAAABBBB genome formula, *B. erectus* is an autotetraploid with an AAAA genome formula and *B. inermis* is auto-allo-octoploid with AAAABBBB genome composition. Armstrong [12], on the basis of the cytological observations, showed that Eurasian species *B. benekenii* (Lange) Trimen and *B. ramosus* Huds. are more closely related to the American species *B. ciliatus* L., *B. latiglumis* Hitchc., *B. pacificus* Shear and *B. richardsonii* Link than to other Eurasian species, and suggested that section *Pnigma* may contain two distinct groups of species with different chromosome size. He also suggested [13] that the large (predominantly Eurasian) and small (predominantly American) chromosome species have followed different evolutionary pathways. Chromosome pairing suggested [13] that the *B. variegatus* Bieb. genome was differentiated from the A and B genomes of octoploid *B. inermis*. All polyploid species of the section, except *B. auleticus*, have identical cpDNA, whereas diploid species of the section showed various degrees of cpDNA divergence [67].

Some species of section *Pnigma* have economic significance. Among them, *B. inermis* is most important as a pasture and forage plant widely cultivated in Northern Europe, Russia, the USA and Asia. *Bromus inermis* (octoploid,  $8x=2n=56$ ) and *B. riparius* (decaploid,  $10x=2n=70$ ) are the most commonly cultivated perennial brome grasses in North America [104]. They are cross-pollinated species, but self-pollination has been reported for *B. riparius* [34]. Hybrids between the two species have been discovered through RAPD and AFLP markers by Fernandez and Coulman [25]. It has been supposed that diploid *B. riparius* found in Kazakhstan could be a progenitor of the *B. inermis* complex [14].

Pillay and Armstrong [66] examined the inheritance of cpDNA in F1 progeny of interspecific crosses of *B. arvensis* with *B. inermis* and *B. erectus*. No intraspecific cpDNA variability was detected. All the F1 progeny examined exhibited the maternal inheritance of cpDNA.

## Section *Ceratochloa*

Section *Ceratochloa* [84], synonyms: subgenera *Ceratochloa* and genera *Ceratochloa*, is a small section in the genus *Bromus*, consisting of 16 polyploid perennial and annual species [15, 67, 100]. No diploid or tetraploid species were found in this section. The section *Ceratochloa* has

the smallest (most primitive) genome size in the genus [15]. The species of section *Ceratochloa* break up into two morphologically distinct groups: the *B. catharticus* hexaploid complex, which is endemic to South America, and the *B. carinatus* octoploid complex, found mainly in North America [98, 67]. All species of subgenus *Ceratochloa* display identical cpDNA sequences. The *B. carinatus* complex appears to be phylogenetically closely related to the diploid *B. anomalus* of section *Phigma* [67]. The six octoploid species of the *B. carinatus* complex are considered to be intersectional amphidiploids between diploids of sect. *Bromopsis* and hexaploid species of sect. *Ceratochloa* [99, 15]. The hexaploid species of section *Ceratochloa* are all found to be strict allopolyploids with genomic formula AABBCC [101].

*Bromus catharticus* is a native of South America and is a predominantly cross-pollinated hexaploid ( $2n=42$ ) species. Puecher et al. [70], using RAPD and AFLP markers, demonstrated great genetic similarity between the morphologically contrasting populations of the *B. catharticus* in Argentina.

In addition, genus *Bromus* also includes three small monospecific sections: (1) section *Nevskiella* contains a single diploid species *B. gracillimus* distributed in Central Asia, Iran and Afghanistan, (2) section *Neobromus* contains a single polyploid species *B. trinii* distributed along the Pacific coast of North, Central and South America, and (3) section *Boissiera* with the only diploid species *B. pumilio*. *Bromus pumilio* is native in Central and Southwest Asia and eastern Mediterranean area. *Bromus pumilio* of the monotypic genus *Boissiera* was firstly considered [83] to belong to section *Bromus*, then was placed in its own section *Boissiera* [90]. The placement of *B. pumilio* in its own section *Boissiera* was supported by allozyme study [60]: this taxon has a basal position in a separate clade in both cladistic and phenetic analyses. This is in accordance with its divergence by morphology and dispersal mechanism [90].

The phylogenetic relationships between these small sections of the genus *Bromus* are obscure and still waiting for investigation. Already in 1981, Stebbins [100] had admitted that genus *Bromus* "is a favourable object for learning more about the evolution of grasses" and it still offers this opportunity. Recently many authors have studied genetic relationships and variation of many of the species of the genus *Bromus*. The results obtained from different molecular markers highlighted the problematic issues for future investigation.

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# **PLANT GENOME: BIODIVERSITY AND EVOLUTION**

Volume 1, Part D

**Phanerogams (Gymnosperm) and  
(Angiosperm-Monocotyledons)**

# Phylogeny and Evolution of *Festuca* L. and Related Genera of Subtribe Loliinae (Poeae, Poaceae)

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PILAR CATALÁN

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## ABSTRACT

*Festuca* and the related grasses of subtribe Loliinae, are major temperate and cool-season forage and grassland species of the world. A review of the evolutionary history of the festucoids, based on recent phylogenetic evidence and on compiled data from different phenetic, genomic and molecular sources, is presented in this chapter. *Festuca* is resolved as a large paraphyletic assemblage with several genera (*Lolium*, *Vulpia*, and others) included within it. Morpho-anatomical, cytogenetic, hybridization and reproductive biological traits of *Festuca* and its close allies support an evolutionary trend from ancestral broad-leaved taxa with large genome sizes and little heterochromatin content, to more recently evolved fine-leaved taxa with small-sized genomes but heterochromatin-rich. Major heterochromatin losses seem to be correlated with the origin of some putatively derived annual lineages. Evolutionary rates differ significantly between the slow-evolving perennial lineages and the rapidly mutating annual lineages, indicating a release from stabilized selection. Hybridization, polyploidy and the acquisition of the annual habit have played a key role in the speciation processes at different evolutionary times. Allopolyploidy emerges as the most widespread mechanism for speciation across the festucoid lineages. Biogeographical analyses indicate a likely late

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Tertiary origin of the diploid Loliinae in Eurasia, followed by successive colonizations and secondary polyploid radiations in the southern hemisphere accompanied by occasional transcontinental long-distance dispersal events. A more recent Quaternary origin is hypothesized for the high polyploid lineages that successfully colonized newly deglaciated areas. Phylogeographical profiles indicate a likely expansion of some Mediterranean and European fescues and ryegrasses in the Holocene-Neocene times.

**Key Words:** Cytogenetics and hybridization, evolution, *Festuca*, Loliinae, molecular and morphological phylogenies

**Abbreviations:** Ca. = Circa, p.p. = pro parte, s.l. = sensu lato, spp = species (plural)

## INTRODUCTION

*Festuca* L. and its related genera form the subtribe Loliinae Dumort., one of the main lineages of the subfamily Pooideae. The most recent phylogenetic studies have revealed that the festucoids are monophyletic and that *Festuca*, as traditionally circumscribed, is not a natural genus, but a large paraphyletic assemblage of distinctly related lineages, with *Lolium*, *Vulpia* and several other genera included within it [28, 29, 77]. The evolutionary study of the festucoids is of great relevance as they represent one of the largest and most widely spread groups of temperate grasses of both ecological and economical importance.

*Festuca* is one of the largest genera of the grass tribe Poeae, accounting for more than 500 species distributed in all continents except Antarctica [92, 169]. The genus is widely distributed across the northern hemisphere and in grassland communities of the southern hemisphere, but restricted to higher altitudes in subtropical and tropical regions [39, 169]. *Festuca* species show the typical pooid-like spikelet with short glumes, several florets, and five-veined lemmas [39, 99, 166]. *Festuca* consists of herbaceous perennial plants, predominantly allogamous, with or without clonal reproduction [92]. The genus is characterized by having species with panicle inflorescences, spikelets with subequal glumes and dorsally rounded lemmas, and caryopses with linear hilums [60]. These species are highly variable in both vegetative and reproductive traits that have traditionally been used to separate them into the 'broad-leaved' fescues, and the 'fine-leaved' fescues [60, 92, 126].

*Festuca* includes some of the most valuable forage grasses of cold and temperate climates of the northern hemisphere, like the meadow and tall fescues [*Festuca pratensis* Huds. and *F. arundinacea* Schreb. complex of *Festuca* subgen. *Schedonorus* (P. Beauv.) Peterm.], and the 'red' and 'ovina' fescues (*F. rubra* L. and *F. ovina* L. groups of *Festuca* subgen. *Festuca*). Montane species of *Festuca* have been used in the restoration of subalpine landscapes and ski slopes (*F. eskia* Raymond ex DC. and *F. gautieri* (Hack.) K. Richt. of *Festuca* subgen. *Festuca*), whereas other fescues are widely planted as ornamentals in gardens (*F. glauca* Vill. group of *Festuca* subgen. *Festuca*). The extensive worldwide ecological range covered by *Festuca* taxa and their abundance in some mountain ecosystems have been used to characterize several grassland phytocenological alliances dominated by fescues [118].

Taxonomic circumscription of *Festuca* and its close allies has changed over the last few centuries (Table 1). *Festuca* is a complex genus divided into several subgenera and sections [4, 5, 6, 7, 9, 60, 61, 62, 93, 94, 115, 165]. Conversely, several segregates of *Festuca*, which were included within this genus in the past (*Vulpia* C. C. Gmel., *Schedonorus* P. Beauv., *Drymochloa* J. Holub, and *Leucopoa* Griseb.), have been recognized as independent genera at different times [43, 58, 70, 71, 139, 140, 143, 167, 168]. One of the most comprehensive studies of *Festuca* was that by Hackel [60], who divided the European fescues into six sections based on characters associated with leaf vernation, leaf sheath, auricles, spikelets and floral bracts (lemma and palea), ovary hairiness and adherence of caryopsis to palea, among others. He also separated infrasectional groups (series) based on the type of shoot innovation and established the anatomical analysis of the leaf cross-sections as a useful approach to identify species and infraspecific taxa [60]. Later, this system was broadly accepted by festucologists [4, 5, 6, 7, 9, 60, 61, 62, 93, 94, 115, 165], who further divided the genus into several subgenera and sections. The most recent revisions of the world's fescues [4, 5, 6, 7, 8, 9] recognized 11 subgenera and several sections within each.

Nine of Alexeev's subgenera [*Festuca* subgenera. *Asperifolia* E.B. Alexeev, *Drymanthele* V. Krecz. & Bobrov, *Erosiflorae* E.B. Alexeev, *Leucopoa* (Griseb.) Hack., *Mallopetalon* (Döll) E.B. Alexeev, *Schedonorus* (P. Beauv.) Peterm., *Subulatae* (Tzvelev) E.B. Alexeev, *Subuliflorae* E.B. Alexeev, and *Xanthochloa* (Krivot.) Tzvelev] are assigned to the broad-leaved fescues, whereas two of them (*Festuca* subgen *Festuca* and *Helleria*

**Table 1.** Taxonomic treatments adopted by different authors for *Festuca* and other related genera of subtribe Lolinae

Hackel (1882-1906)	Tzvedev (1971-2000)	Cotton & Stace (1977) Stace (1981)	Alexeev (1977-1986)	Kerguelen & Plonka (1989)	Clayton & Ranvoize (1986)	Holub (1998)	Catalán et al. (2004) Müller & Catalán (2005)
<i>Festuca</i>	<i>Festuca</i>	Stace (1981)	<i>Festuca</i>	<i>Festuca</i>	<i>Festuca</i>	<i>Festuca</i>	<i>Festuca</i>
subgenus	subgenus <i>Festuca</i>		subgenus <i>Festuca</i>	subgenus <i>Festuca</i>			subgenus <i>Festuca</i> <sup>a</sup>
Eu <i>Festuca</i>	sect. <i>Festuca</i>						
sect. <i>Oviniae</i>							sect. <i>Festuca</i> <sup>a</sup>
- <i>Intravaginales</i>				sect. <i>Festuca</i>			sect. <i>Aulaxyper</i> <sup>a</sup>
- <i>Extravaginales</i>				sect. <i>Aulaxyper</i>			
vel <i>mixtae</i>							
sect. <i>Variiae</i>	sect. <i>Variiae</i>			sect. <i>Eskia</i>			sect. <i>Eskia</i> <sup>a</sup>
				sect. <i>Amphigenes</i>			sect. <i>Dimorphuae</i> <sup>a</sup>
sect. <i>Subbulbosae</i>				sect. <i>Subbulbosae</i>			sect. <i>Subbulbosae</i> <sup>b</sup>
							sect. <i>Lojaconoa</i> <sup>b</sup>
							subgenus <i>Helleria</i> <sup>a</sup>
sect. <i>Bovinae</i>	subgenus <i>Schedonorus</i>		subgenus <i>Helleria</i>	subgenus <i>Schedonorus</i>		<i>Schedonorus</i>	subgenus <i>Schedonorus</i> <sup>b</sup>
	sect. <i>Subulatae</i>		subgenus <i>Schedonorus</i>	subgenus <i>Subulatae</i>			subgenus <i>Subulatae</i> <sup>ab</sup>
			subgenus <i>Drymanthele</i>	subgenus <i>Montanae</i>		<i>Drymanchloa</i>	subgenus <i>Drymanthele</i> <sup>b</sup>
sect. <i>Montanae</i>							sect. <i>Scariosae</i> <sup>b</sup>
sect. <i>Scariosae</i>							sect. <i>Pseudoscartosae</i> <sup>b</sup>
	subgenus <i>Leucopoa</i>		subgenus <i>Leucopoa</i>	subgenus <i>Hesperochloa</i>		<i>Leucopoa</i>	subgenus <i>Leucopoa</i> <sup>ab</sup>
	sect. <i>Leucopoa</i>		subgenus <i>Subuliflorae</i>	subgenus <i>Subuliflorae</i>			
	sect. <i>Amphigenes</i>		subgenus <i>Obtusae</i>	subgenus <i>Obtusae</i>			
	sect. <i>Brevistriatae</i>		subgenus <i>Erosiflorae</i>				
			subgenus <i>Mallopetalon</i>				
	subgenus <i>Xanthochloa</i>		subgenus <i>Xanthochloa</i>	subgenus <i>Xanthochloa</i>			

Table 1 contd.

Table 1 contd.

subgenus <i>Vulpia</i>	<i>Vulpia</i>	<i>Vulpia</i>	<i>Vulpia</i>	<i>Vulpia</i>	<i>Vulpia</i>	<i>Vulpia</i> <sup>a</sup>
	sect. <i>Vulpia</i>					
	sect. <i>Monachme</i>					
	sect. <i>Spirachme</i>					
	sect. <i>Loretia</i>					
	sect. <i>Apalochloa</i>					
<i>Ctenopsis</i>	<i>Ctenopsis</i>				<i>Ctenopsis</i>	<i>Ctenopsis</i> <sup>a</sup>
<i>Microphyllum</i> ,	<i>Microphyllum</i>				<i>Microphyllum</i>	<i>Microphyllum</i> <sup>a</sup>
	<i>Narduroides</i>					<i>Narduroides</i> <sup>a</sup>
	<i>Psilurus</i>				<i>Psilurus</i>	<i>Psilurus</i> <sup>a</sup>
<i>Wangenheimia</i> ,	<i>Wangenheimia</i>				<i>Wangenheimia</i>	<i>Wangenheimia</i> <sup>a</sup>
<i>Castellia</i>		<i>Lolium</i>		<i>Lolium</i>	<i>Castellia</i>	<i>Castellia</i> <sup>b</sup>
					<i>Lolium</i>	<i>Lolium</i> <sup>b</sup>
					<i>Microphyllum</i>	<i>Microphyllum</i> <sup>b</sup>

Abbreviations: <sup>a</sup>Fine-leaved Festuca; <sup>b</sup>Broad-leaved Festuca.

E.B. Alexeev) are circumscribed to the fine-leaved fescues [4, 5, 6, 7, 8, 9] (Table 1). *Festuca* subgenus *Drymanthele* has been considered to be of relict origin, reflected by their putatively primitive leaf-blade and panicle features [60, 70, 165]. It encompasses the tallest extant *Festuca* species, which bear remarkably wide leaf-blades, large culms and panicles, and live in forested habitats of the Holarctic region. By contrast, the remaining broad-leaved subgenera show more restricted geographical distribution patterns and mostly grow in more mesic ecosystems. The fine-leaved fescues have been traditionally interpreted as more recently evolved taxa [60, 70, 165] because of their slender habit and successful adaptation to a range of humid to xeric habitats. *Festuca* subgenus *Festuca* encompasses the broad *Festuca* sections *Festuca* (*F. ovina* group) and *Aulaxyper* Dumort. (*F. rubra* group), together accounting for the highest number of described species in the genus. These are widely distributed across the Holarctic region and in the highest altitudes and latitudes of the southern hemisphere. These large sections share several morpho-anatomical traits, including grains adnate to the paleas, short truncate ligules, and conduplicate innovation leaves [60, 162].

*Lolium* L. is separated from *Festuca* in most current floras, based on distinctive inflorescence traits, particularly spikelets sunk in the excavated rachis of the spike, each covered by a single glume, and a lemma with more than five veins [159]. *Lolium* encompasses ca. 10–12 species that are mostly native to the pan-Mediterranean region from the Middle East to Macaronesia. *Lolium* taxa show a trend from short perennials/biennials to annuals, and from allogamous to autogamous plants, though no infrageneric ranks have been recognized for them [159]. The genus includes species of high economic importance, like the perennial and Italian ryegrasses (*Lolium perenne* L. and *L. multiflorum* Lam.), extensively used for grazing, turf and amenity purposes, and the cereal weed *Lolium rigidum* Gaudin. The affinity between representatives of *Lolium* and *Festuca* subgenus *Schedonorus* has been demonstrated by spontaneous and artificial intergeneric crosses [80, 97], similarity in chromosome banding patterns [160], and a series of molecular studies on seed-proteins, plastid restriction sites, nuclear RFLP (restriction fragment length polymorphism) and RAPD (random amplified polymorphic DNA) markers, and nuclear and plastid DNA sequence analysis [2, 25, 28, 35, 55, 76, 96, 146]. *Lolium* also shares morphological traits with *Festuca* subgenus *Schedonorus*, such as the possession of falcate

auricles. This character is also present in *Micropyropsis tuberosa* Romero-Zarco & Cabezudo, a monotypic, short lived perennial endemic to the western Mediterranean region [122], and in *Castellia tuberculosa* (Moris) Bor, a monotypic annual that occurs in the pan-Mediterranean–Asian area [39, 169].

*Vulpia* was erected as a new genus [85] and has been separated from *Festuca* since based on their annual habit, very unequal glumes, and long-awned lemma though none of these characters is absolute [43]. Up to five different sections have been recognized within *Vulpia* [*Apalochloa* (Dumort.) Stace, *Loretia* (Duval-Jouve) Boiss., *Monachne* Dumort., *Spirachne* (Hack.) Boiss., and *Vulpia*] (Table 1), based on breeding system, spikelet structure, and several floral traits [43, 141, 143]. *Vulpia* taxa show a trend towards reduction in size and increasing self-fertility through sections *Loretia*, *Monachne/Spirachne* and *Vulpia* [43]. *Vulpia* accounts for ca. 22 species native to the Mediterranean region and America; the chromosome number is fixed within each *Vulpia* species and shows a geographical distribution pattern [42]. Representatives of three sections of *Vulpia* (*Vulpia*, *Monachne* and *Loretia*) hybridize with representatives of *Festuca* section *Aulaxyper* (*Festuca rubra* complex) in the wild or in artificial crosses [3, 15] and show affinities in chromosome pairing [11].

Another 11 annual genera (*Castellia* Tin., *Catapodium* Link, *Ctenopsis* De Not., *Cutandia* Willk., *Desmazeria* Dumort., *Lolium* Krecz. & Bobr., *Micropyrum* Link, *Narduroides* Rouy, *Sclerachloa* P. Beauv., *Vulpiella* (Batt. & Trab.) Burollet, and *Wangenheimia* Moench) mostly native to the Mediterranean region were grouped with *Vulpia* in the *Vulpia-Desmazeria* complex [143]. These genera were ranked as being allied to *Vulpia*, *Desmazeria*, or as an intermediate among them [143]. These genera plus *Psilurus*, a further monotypic isolated Mediterranean genus, are differentiated from one another by their leaf-blade characteristics, inflorescence type, floral and hilum length, and were considered to merit generic status [39, 141, 142] (Table 1). *Festuca* and its satellite genera constitute one of the three main Poae lines. Clayton and Renvoize [39] suggested that Mediterranean annuals evolved from mountain-grassland perennials. Classical circumscriptions of the festucoids placed from nine to 15 genera in subtribe Festucinae C. Presl [39, 166]; nomenclatural priority favours Loliinae Dumort. over Festucinae [49, 137] as the correct subtribe name for the festucoids.



Festucoids have been subjected to repeated taxonomic splitting and lumping. The recent advent of molecular phylogenetics has affected traditional classifications in this group and that of close subtribes and genera in the tribe Poeae R. Br. [27, 45, 72, 137]. However, unexpected evolutionary relationships have been revealed and previous hypotheses have been reassessed from our molecular-based cladistic and Bayesian analyses [28, 29, 77, 162, 164]. Molecular phylogenetics have also provided a solid evolutionary framework to test evolutionary hypotheses related to speciation processes involving genome rearrangements, differential mutation rates, hybridization, and polyploidization [28, 30, 164]. In addition, molecular phylogenies allow speculation on life history traits connected with extinctions, migration and radiation events reflected in the biogeographical and phylogeographical patterns [14, 52, 76, 77]. A series of studies conducted at both macroevolutionary and microevolutionary scales have devised new scenarios for understanding the origin and divergence of the festucoid lineages and allies [30, 31, 77].

## CLASSICAL AND MODERN CIRCUMSCRIPTIONS OF THE FESTUCOIDS

As with many studies of angiosperms [67], the phylogeny of subtribe Loliinae revealed by nuclear and plastid data sets does not totally agree with traditional classifications based on morphological traits [4, 5, 6, 7, 8, 9, 60, 166]. However, some of the resolved lineages presented in the successive phylogenetic studies of *Festuca* and allies [28, 29, 76, 77, 162, 164] are more or less concordant with previous taxonomic circumscriptions for these groups (Table 1; Fig. 1). Nonetheless, systematic classifications remain unclear because of the large paraphyly found within *Festuca*, the instances of incongruence detected between molecular and morphological data, and the uncertain ascription of several newly described species of *Festuca* from the American, Asian and Austro-New Zealand continents [36, 41, 147, 148, 149, 150, 151].

Recent proposals suggest different scenarios for the classification of the festucoids [29] ranging from a synthetic monophyly criterion that would favour the treatment of all Loliinae taxa as members of a large and morphologically diverse genus *Festuca*, to many smaller generic and infrageneric splits. One of these includes an evolutionary systematics-based criterion that is nomenclaturally conservative and that recognizes both monophyletic and paraphyletic groups (i.e. *Lolium*, *Schedonorus*) as

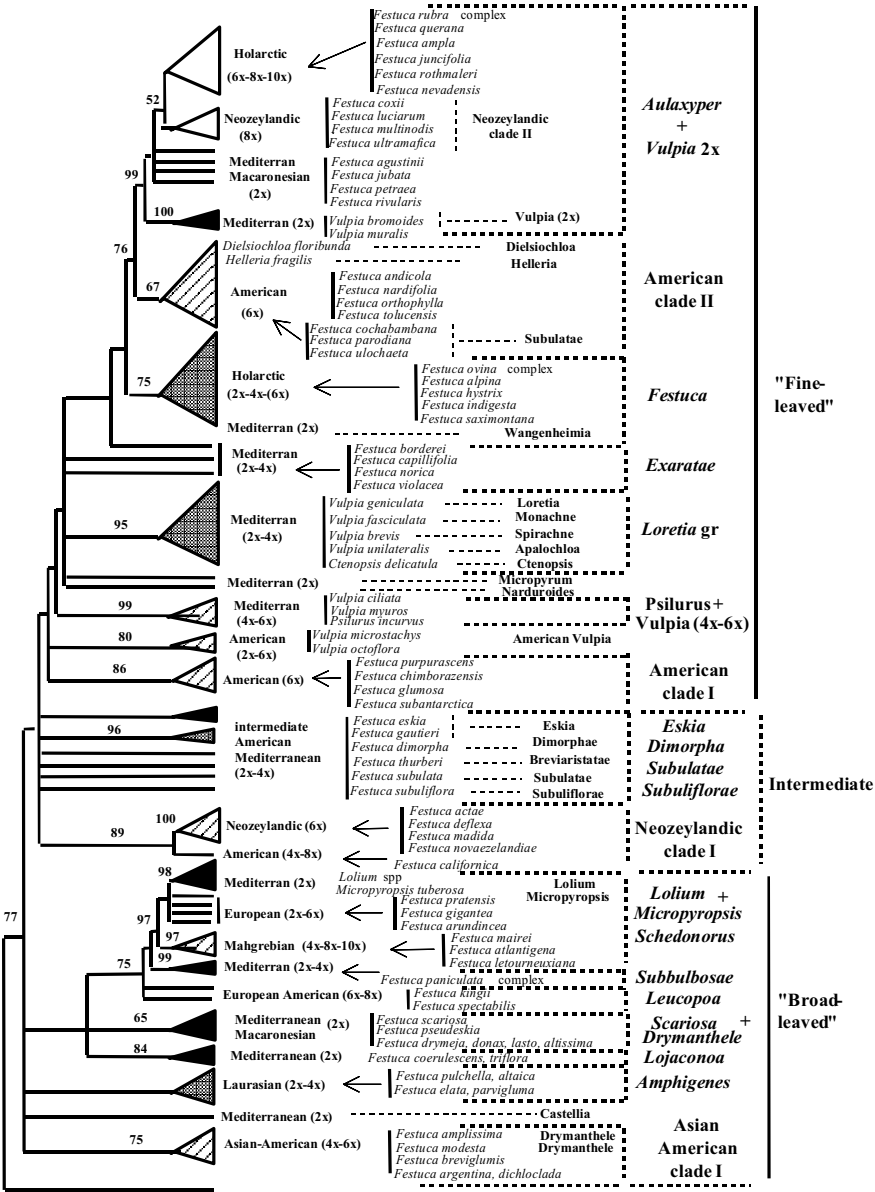


Fig. 1. Summarized cladistic tree of the festucoids (subtribe Loliinae) based on combined analysis of ribosomal ITS and plastid trnTF sequences (taken from Catalán et al. [28], Inda et al. [77], and unpublished data). Numbers above branches correspond to bootstrap values. Geographic distribution and ploidy levels are indicated for the main lineages (ploidy increases from black (2x) to white (8x-10x) clades).

independent taxonomic entities [29]. This criterion was applied in recent floras by those authors who recognize some of the broad-leaved lineages of Loliinae as unique genera outside of *Festuca* [140].

Plastid RFLP data [44] and ribosomal ITS sequences [35, 55, 162] first demonstrated that *Festuca* was paraphyletic and that *Lolium* and *Vulpia* were nested within *Festuca*. The closeness of *Lolium* to the broad-leaved *Festuca* of subgenus *Schedonorus* and that of *Vulpia* to the fine-leaved *Festuca* confirmed previous findings based on chromosome analyses and on artificial crosses [3, 15, 80, 100]. After combined analysis of chloroplast restriction sites and structural characters, *Catapodium*, *Cutandia* and *Desmazeria* were classified as belonging to a separate lineage, subtribe Parapholiniinae [137].

Further investigations on the relationship of *Festuca* and related genera based on independent and simultaneous analyses of nuclear and plastid DNA sequences have extended the range of paraphyly of both the broad and fine-leaved fescues [28, 29, 77, 164] (Fig. 1). The most exhaustive molecular studies have found a likely evolutionary trend from more ancestral broad-leaved *Festuca* lineages towards more recently derived fine-leaved ones [28, 77]. Polyphyletic *Vulpia* and other Mediterranean genera of ephemerals (*Ctenopsis*, *Micropyrum*, *Narduroides*, *Psilurus* and *Wangenheimia*), which are nested within the fine-leaved *Festuca* clade, American perennials *Helleria* and *Dielsiochloa*, a genus previously attributed to Aveneae [39], also fall within the fine-leaved group, whereas *Lolium* and *Micropyropsis* are included within the broad-leaved clade (Fig. 1). Previous results indicate that the sister clade Dactylidinae Stapf, which includes the orchard grass genus *Dactylis* L. plus *Lamarckia* Moench, and the Cynosurinae Fr./Parapholiniinae Caro (*Catapodium*, *Cutandia*, *Cynosurus* L., *Desmazeria*, *Hainardia* Greuter, *Parapholis* C. E. Hubbard and *Sphenopus* Trin.), are the closest relatives of Loliinae [28, 77]. Affinity of *Cutandia* to the Parapholiinae has been recently confirmed through further genome sequence analysis [77], solving previous misattributions of this genus to the *Vulpia* sect. *Loretia* 'assemblage' [28, 164].

A larger taxon sampling helped to illustrate the evolutionary history of the main *Festuca* lineages and relatives [29, 77, 111]. These studies concur with the previous hypotheses on the evolutionary trend from more ancestral broad-leaved *Festuca* lineages (subgenus *Drymanthele* + section *Scariosa* Hack. + section *Pseudoscariosa* Krivot., section *Lojaconoa* Catalán & Müller) towards less ancestral broad-leaved groups (section

*Subbulbosae* Nyman, subgenus *Leucopoa*, subgenus *Schedonorus* + *Micropyropsis* + *Lolium*), and then to the more recently evolved crown group of fine-leaved festucoids (Fig. 1). However, the range of morphologically broad to intermediate size leaved lineages has been extended considerably within this last group, with several *Festuca* lineages (subgenus *Subulatae* p.p., subgenus *Subuliflorae*, section *Dimorphae* Müller & Catalán, plus narrow-leaved section *Eskia* Willk.) aligned basally to the fine-leaved clade. The successive divergences of lineages of the *Psilurus* + polyploid *Vulpia* section *Vulpia* group, the annual 'Loretia assemblage' (*Vulpia* section *Loretia* + *Monachne* + *Spirachne* + *Apalochloa* plus *Ctenopsis*), and *Festuca* subsection *Exaratae* ultimately led to the most recently evolved *Festuca* section *Aulaxyper* + diploid *Vulpia* section *Vulpia* and *Festuca* section *Festuca* + *Wangenheimia* core clades (Fig. 1).

Geographic structure has been detected within the *Festuca* subgenus *Schedonorus* group [28, 76]. This group is separated into two subgroups: (1) a 'European' subclade that encompasses diploid *Festuca fontqueri* St.-Yves and *F. pratensis*, and hexaploid *F. arundinacea* and *F. gigantea* (L.) Vill., with *Micropyropsis* and diploid *Lolium* derived from within it, and (2) a 'Maghrebian' subclade, which includes tetraploid western Mediterranean *F. fenas* Lag. and *F. mairei* Hack. ex Hand.-Mazz. and their high polyploid North African derivatives (*F. arundinacea* var. *atlantigena* (St.-Yves) Auquier and *F. arundinacea* var. *letourneuxiana* Torrecilla & Catalán).

More exhaustive phylogenetic surveys using both nuclear and plastid genomes indicate a biogeographical pattern resulting in New Zealand and American clades of various taxonomic ranks that are distinctly resolved at intermediate placement between the broad and fine-leaved lineages (Neozeylandic clade I) and within the fine-leaved group (American clades I and II) (Fig. 1), paralleling the cases found in the large genus *Poa* L. (Poinae, Poeae) of bluegrasses [136]. The *Aulaxyper* clade is enlarged by several putatively relict, diploid Macaronesian elements (*Festuca agustinii* Linding., *F. jubata* Lowe, *F. petraea* Guthnick) that align basally with another Mediterranean diploid congener (*F. rivularis* Boiss.), and by another polyploid Neozeylandic (clade II) close to the Holarctic high polyploid clade (Fig. 1). Also an American *Helleria* + *Dielsiochloa* + *Festuca* subgenus *Subulatae* p.p. clade shows a close relationship to the red fescues [77] (Fig. 1).

The most updated studies indicate that *Lolium* and *Vulpia* have had different life histories and origins [76, 77]. The diploid and

morphologically homogeneous ryegrasses probably diverged recently as they are resolved as monophyletic and derived from a European *Festuca* subgenus *Schenodorus* lineage. By contrast, the more variable and geographically widespread diploid to polyploid *Vulpia* species show a larger extended polyphyly and incompletely resolved relationships within one another, and to other fine-leaved *Festuca* lineages. Perhaps this is a result of a more ancestral and complex radiation pattern with several polytopic origins [77] (Fig. 1).

Other geographically isolated genera (*Austrofestuca* E. B. Alexeev, *Parafestuca* E. B. Alexeev), formerly classified within *Festuca* and more recently separated from it, have been found to be unrelated to *Festuca* and to the festucoids [39]. Molecular phylogenetic reconstructions have resolved Australian and New Zealand *Austrofestuca* as members of a core Poinae clade [56, 75], whereas the Macaronesian *Parafestuca* falls within a core Aveneae clade [116].

Current investigations on evolutionary relationships of Loliinae, within a broad supratribal Aveneae-Poeae framework, have shaped the monophyly of this lineage and its relative isolation with respect to the pooid and avenoid clades [31]. Recent studies have detected potential horizontal gene transfer of mitochondrial *Festuca* sequences into the less related genomes of *Secale* (Triticeae) and *Danthonia* (Danthonieae) (H. C. Ong, S. Chang, and J. D. Palmer, personal communication).

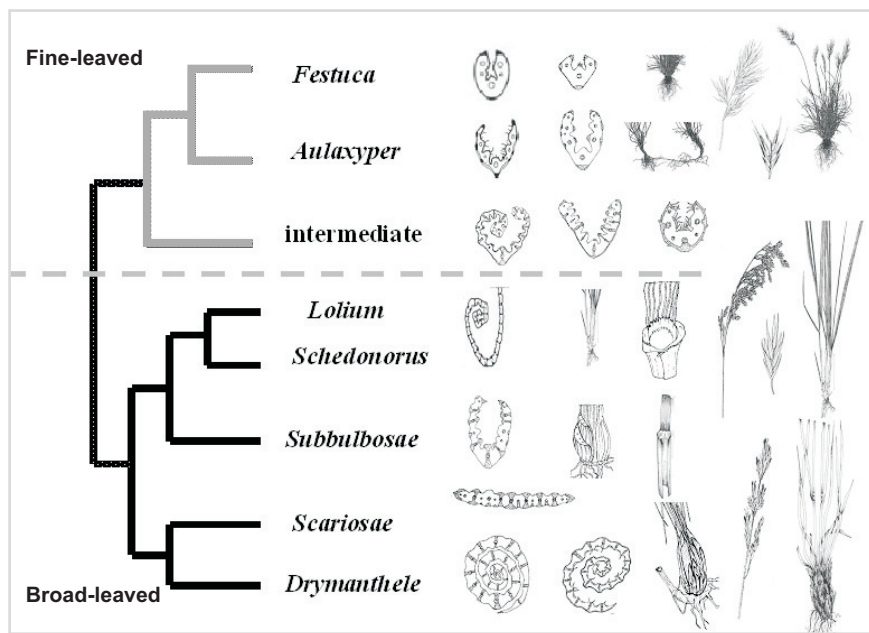
## CONFLICTS AND CONCERTS BETWEEN MOLECULES AND MORPHOLOGY IN THE LOLIINAE

Conflicts between the Loliinae topologies recovered from different genomes have been interpreted as a consequence of interacting phenomena, such as lineage sorting or reticulation [28]. The existence of past hybridization events might obscure phylogenetic reconstruction within the temperate grasses [45, 91, 105, 137]. Chloroplast capture, allopolyploidy and lineage sorting are hypotheses invoked to explain the failure to reconcile topologies recovered from different genomes in Triticeae [91, 105] and these have been advocated to interpret the unexpected placements of some *Vulpia* and other ephemeral taxa within the fine-leaved clade and of *Festuca* subgenus *Leucopoa*, subgenus *Subulatae*, and section *Amphigenes* s.l. across the broad and fine-leaved clades of the festucoids [28, 77]. Paralogy might be another disturbing factor that affects phylogenetic reconstruction within a single data set,

since pseudogene copies of ITS sequences have been detected in *Lolium* [55] and can be extended to other festucoid lineages [164].

Structural characters are believed to have arisen through different gene regulatory mechanisms or developmental pathways in grasses [84, 137] and, therefore, would be expected to be congruent with molecular phylogenies. However, discrepancies in phylogenetic reconstruction between molecular and structural evidence are frequent within Poaceae [59]. Incongruence between molecular-based and morphology-based approaches has been related to the innate plasticity of morphological features and their consequent higher homoplasy that makes them inappropriate to recover deep phylogenetic signals in macroevolutionary groups, such as the subfamily Pooideae [90]. A careful examination of a large set of morphological traits within Loliinae and close subtribes (primary synapomorphies, sensu de Pinna [47]) led to the discovery of several secondary synapomorphic characters that support more inclusive groups [29] (Fig. 2). These traits correspond to changes in qualitative characters that mark preferentially the deeper nodes of the tree and that could be associated with heterotopic changes, resulting from shifts in the expression of developmental pathways in different plant organs, as indicated by Kellogg [84].

Incongruences between molecules and morphology mostly affect the resolution of the broad-leaved *Festuca* lineages, some fine-leaved *Festuca*, and the *Vulpia* lineages [29]. Among them, the Holarctic subgenus *Drymanthele*, the Asian-North American subgenera *Leucopoa* and *Subulatae*, the European section *Amphigenes*, and the Western Mediterranean section *Subbulbosae* are resolved as polyphyletic taxa. The Eurasian and Mediterranean subgenus *Schedonorus* appears to be paraphyletic with *Lolium* and *Micropyropsis* included within it (Fig. 1). *Festuca* subgenera *Drymanthele*, *Leucopoa*, and *Subulatae*, as well as section *Amphigenes* share several foliar characters related to the 'broad-leaved' syndrome. *Leucopoa* (section *Leucopoa*) differs from the others in being dioecious. *Festuca* sections *Subbulbosae*, *Lojaconoa*, *Scariosae*, and *Pseudoscariosa* show intermediacy in their characters. Another striking finding is the wide polyphyly shown by species of the 'transclade' subgenera *Leucopoa* and *Subulatae*, with taxa exhibiting broad-leaved traits but nested both at the base of the fine-leaved clade and close to the *F. rubra* group clade (Fig. 1). The artificiality of various broad-leaved hierarchic ranks (i.e. *Festuca* subgenera *Subulatae* and *Drymanthele*) was already manifested in previous studies [28, 36, 44].



**Fig. 2.** Evolution of morphological characters across several representatives of broad-leaved (subgenus *Drymanthele*, section *Scariosae*, subgenus *Schedonorus* + *Lolium*), intermediate (section *Eskia*, section *Dimorphae*, subgenus *Leucopoa* p.p.), and fine-leaved (sections *Aulaxyper* and *Festuca*) Loliinae lineages. Branch darkness indicates character-states associated with the 'broad-leaved syndrome' (flat leaves, sclerenchyma trabeculate, extravaginal shoots, cataphylls present, convolute to supervolute veneration), branch lightness indicates those associated with the 'fine-leaved syndrome' (thin leaves, sclerenchyma non-trabeculate, intravaginal (and mixed) innovation shoots, cataphylls mostly absent, plicate veneration).

The most recently derived *Festuca* sections *Festuca* and *Aulaxyper* are resolved as monophyletic and with moderate support for groups that include the type species and closest relatives (Fig. 1). The most unexpected resolution is that obtained for *Vulpia*, which shows a high polytomy for members of typical section *Vulpia*, with species taxonomically similar to each other but with chromosome and geographical races distinctly related to the red-fescues (Mediterranean diploids), to the American subclade of fine-leaved fescues [American *V. octoflora* (Walter) Rydb. and *V. microstachys* (Nutt.) Munro], or in an unresolved but supported Mediterranean polyploid clade with *Psilurus* (tetraploids, hexaploids). A further resolved but differently supported



lineage is that of representatives of *Vulpia* sections *Loretia*, *Monachne*, *Spirachne*, *Apalochloa* plus *F. plicata* Hack.; which also incorporates *Ctenopsis* (Fig. 1). Relationships of other annual genera, i.e. *Micropyrum*, *Wangenheimia*, *Narduroides* are not satisfactorily resolved as they show distinct and poorly supported relationships in nuclear vs. plastid trees [28, 77].

Simultaneous cladistic analysis of morphological and molecular data within Loliinae and close subtribes was conducted to evaluate the phylogenetic signal of selected morpho-anatomical traits [37, 43, 60, 90, 94, 102, 103, 125, 141, 143, 159, 169, 171]. This analysis resulted in an overall lack of resolution for the Loliinae and its allies [29]. However, better resolution was obtained when the analysis was restricted to Loliinae representatives and secondary synapomorphies were detected for several lineages. The presence of a long linear hilum was found to be synapomorphic for the festucoids (except for a reversal in *Wangenheimia*). The thickened base of the leaf sheath is shared by *Festuca* sections *Subbulbosae* and *Lojaconoa*. The trend towards reduced spike-type inflorescences, an excavated rachis, and a single glume has evolved at least three times in the *Lolium*, *Psilurus*, and *Hainardia* lineages. The fine-leaved *Festuca* subgenera *Festuca* and *Helleria* share a perennial habit, conduplicate vernation, and an awned lemma. The basal *Festuca* sections *Eskia* and subgenus *Helleria* have common traits, such as a hairy ovary tip, and some of the intermediate *Festuca* subsections *Festuca* and *Exaratae* representatives have leaf blades that possess a continuous abaxial ring of sclerenchyma. The more recently diverged *Festuca* section *Aulaxyper* s. l. group is characterized by an admixture of intravaginal and extravaginal innovation shoots and reduced cataphylls. The *Festuca* subgenus *Schedonorus* + *Micropyropsis* + *Lolium* clade is characterized by falcate auricles and awned lemmas –unique features within the broad-leaved group– whereas representatives of *Festuca* section *Amphigenes* and *Festuca* subgenera *Drymanthele*, *Leucopoa*, and *Subulatae* lack falcate auricles and awned lemmas, but, like *Festuca* subgenus *Schedonorus* + *Micropyropsis* + *Lolium*, present innovation leaves with complete abaxial and adaxial sclerenchyma trabecules. The basalmost groups also bear prominent cataphylls, convolute to supervolute vernation, and creeping extravaginal rhizomes [29] (Fig. 2). These results partly agree with the hypothesis brought forward by Hackel (1882), Holub (1984), and Tzvelez (1982) on the general evolutionary trend from more primitive robust broad-leaved *Festuca* towards more advanced slender fine-leaved



*Festuca* [60, 70, 166]. Interpretation of the character state changes along the broad-leaved *Festuca* clade indicates that the 'broad-leaved' foliar syndrome could be plesiomorphic and could also have evolved several times towards the opposite trend along the clade. The lack of resolution for these, however, and the presence of old lineages of intermediate taxa in most subclades, raised doubts about their ancestry [28].

Concert between molecules and morphology has been evidenced more convincingly at microevolutionary scale in *Festuca* and *Lolium* [13, 14, 51, 163]. Quantitative morphological changes might be postulated to characterize the shallow nodes of the trees in Poeae, as indicated by Kellogg [84]. Highly variable molecular markers and quantitative morphological traits have been useful tools in characterizing the most recently derived lineages of Loliinae. Different allozymic combinations have been found for strains of *Lolium perenne* and *L. rigidum* across Europe [13], and multilocus RAPD phenotypes have detected taxonomic structure among the four polyploid microspecies of the *Festuca brachyphylla* complex in the arctic island of Svalbard [51]. Combined multivariate analysis of RAPD phenotypes and of quantitative morphological traits showed *F. baffinensis* Polunin, *F. brachyphylla* Schult. & Schult. f., *F. hyperborea* Holmen & Fredericksen, and *F. edlundiae* S. Aiken, Consul & Lefkov. to be different genetic entities [51]. The potential value of the highly polymorphic markers (RAPD) fails, however, when recovering genetic affinities of more removed Loliinae groups [35, 146]. Similarly, quantitative morphological traits are useless in phylogenetic reconstructions due to the inherent problems of homology presented by any proposed system of coding of continuous characters [117]. However, phenetic analyses of morphometric characters have provided a baseline to detect taxonomic structure in the Mediterranean species of *Festuca* section *Eskia* [163], and, combined with RAPD data, to clarify the hybrid origin of wild sterile *Festuca* taxa (*F. x picoeuropeana* Nava, *F. x souliei* St.-Yves) from their respective putative parents (*F. eskia* x *F. gautieri* and *F. eskia* x *F. quadriflora* Honck.) in hybrid zones of the northern Iberian and Pyrenean mountain ranges [108, 157].

## **SPECIATION AND EVOLUTIONARY TRENDS IN LOLIINAE: C-VALUES, KARYOTYPE EVOLUTION, HYBRIDIZATION AND POLYPLOIDY**

Cytogenetic and hybridization studies within and among *Festuca*, *Lolium* and *Vulpia* provided the key for the analysis of speciation processes and

evolutionary trends within subtribe Loliinae [3, 11, 12, 20, 46, 48, 63, 73, 78, 79, 80]. These results have been re-interpreted in the light of our present phylogenetic knowledge.

*Festuca* and its close allies show uniformity in the chromosome base number  $x = 7$ , which is otherwise characteristic of Poeae and other tribes of the Pooideae core clade [69, 138]. The lack of aneuploid series on the number of chromosomes per set has been interpreted as evidence of a relatively recent origin of Poeae [27, 72, 138]. However, the large number of ploidy levels reported in *Festuca* and some relatives, i.e. *Vulpia*, was interpreted as a consequence of having an old evolutionary history among different lineages [46, 164]. Ploidy levels range from diploids to duodecaploids in *Festuca* [28, 48, 162] though diploids and polyploids are differentially distributed across the main subgenera and sections. Some of the purportedly oldest broad-leaved *Festuca* lineages consist exclusively (*Drymanthele*, *Lojaconoa*, *Scariosae* and *Pseudoscariosa*) or predominantly (*Subbulbosae*) of diploids. Within the fine-leaved *Festuca*, the basal lineages (*Eskia*, *Dimorpha*) also show higher percentages of diploid taxa. Polyploidy is extensive in *Festuca*, up to 70% [48, 100]. Most polyploids belong to the largest and more recently evolved fine-leaved taxa of *Festuca* subgenus *Festuca*; however, some intermediate and broad-leaved lineages also incorporate diploids and polyploids (*Schedonorus*) or are formed exclusively by distinct polyploid ranges (*Leucopoa*, *Subulatae*) [28].

The evolution of polyploidy has clearly emerged parallel to the main lineages of the broad and fine-leaved *Festuca*, though the mechanisms experienced by each lineage probably occurred at different times. Within the fine-leaved fescues, the two largest groups (*F. ovina* and *F. rubra*) show opposite patterns of ploidy. In the *F. ovina* group, most taxa are diploids or low polyploids (tetraploids), in the red fescues (*F. rubra*), diploids and tetraploids are rare compared to the more abundant hexaploids and higher ploidy level taxa [3]. High ploidy levels also dominate some of the broad-leaved lineages of *Festuca* subgenus *Schedonorus*, i.e. *Festuca arundinacea* complex, and *Leucopoa* [*F. kingii* (S. Watson) Cassidy].

The closest relatives of *Festuca* show differences in chromosome numbers. Within the fine-leaved lineage, most of the ephemeral genera are diploids (*Ctenopsis*, *Micropyrum*, *Nardurioides*, *Wangenheimia*), *Psilurus* is tetraploid and *Vulpia* exhibits three ploidy levels (from diploid to hexaploid) [42, 143]. The relict distribution of these endemic

Mediterranean genera and their low ploidy levels have been interpreted as evidence of ancient origin [164]. The variable ploidy-levels in the geographically widespread species of *Vulpia* suggest a longer evolutionary history. By contrast, within the broad-leaved lineage, diploid *Lolium* species could be considered as diverging recently from a *Schedonorus* lineage that has maintained its diploid level up to the present day [28, 100].

Karyotype evolution in subtribe Loliinae is also concordant with hypotheses recovered from our molecular data (Fig. 3). *Festuca* shows striking differences in genome sizes between the broad and fine-leaved lineages [20, 131, 132]. Among diploid *Festuca* species and their closest allies (*Lolium*, *Vulpia*), there is considerable variation in chromosome size and nuclear DNA quantity [20, 131, 132], showing a trend from the large chromosomes and high C-values of the more ancestral broad-leaved *Drymanthele* and *Scariosae* taxa, through intermediate chromosomes and medium C-values of the more advanced broad-leaved *Schedonorus* and *Lolium* taxa, to the small chromosomes and low C-values of the more recent fine-leaved *Festuca*, *Aulaxyper* and *Vulpia* taxa [20] (Fig. 3). Karyotypic C-banding patterns are highly correlated with this variation, though surprisingly, heterochromatin content is inversely correlated with genome size in *Festuca* and its allies [11, 46, 160]. Cytogenetic analyses demonstrated a 2.5-fold range decrease in chromosome size coupled with a 7.5-range increase in absolute heterochromatin content from primitive broad-leaved *Festuca* lineages (*Drymanthele*, *Scariosae*, *Subbulbosae*) to the more advanced fine-leaved *Festuca* lineages (*Schedonorus*, *Eskia*, *Festuca*, *Aulaxyper*) [11, 46].

A full range of C-banding patterns have been found across the Loliinae lineages [11, 46, 160] (Fig. 3), paralleling other pooid groups such as Triticeae [133] and *Avenae* [121]. Karyotypes of ancestral broad-leaved *Scariosae* and *Subbulbosae* lineages are characterized by near metacentric chromosomes with very little heterochromatin distributed in a few bands (Fig. 3), resembling the *Triticum* D-genome [46]. The ancestral *Drymanthele* lineage shows larger and more unequal chromosomes with a slight increase in heterochromatin resulting in the addition of small centromeric and a few intercalary bands [46] (Fig. 3). The more advanced *Schedonorus* and *Lolium* lineages have smaller submetacentric chromosomes and intermediate levels of heterochromatin distributed among centromeric and intercalary bands [46, 160] (Fig. 3),

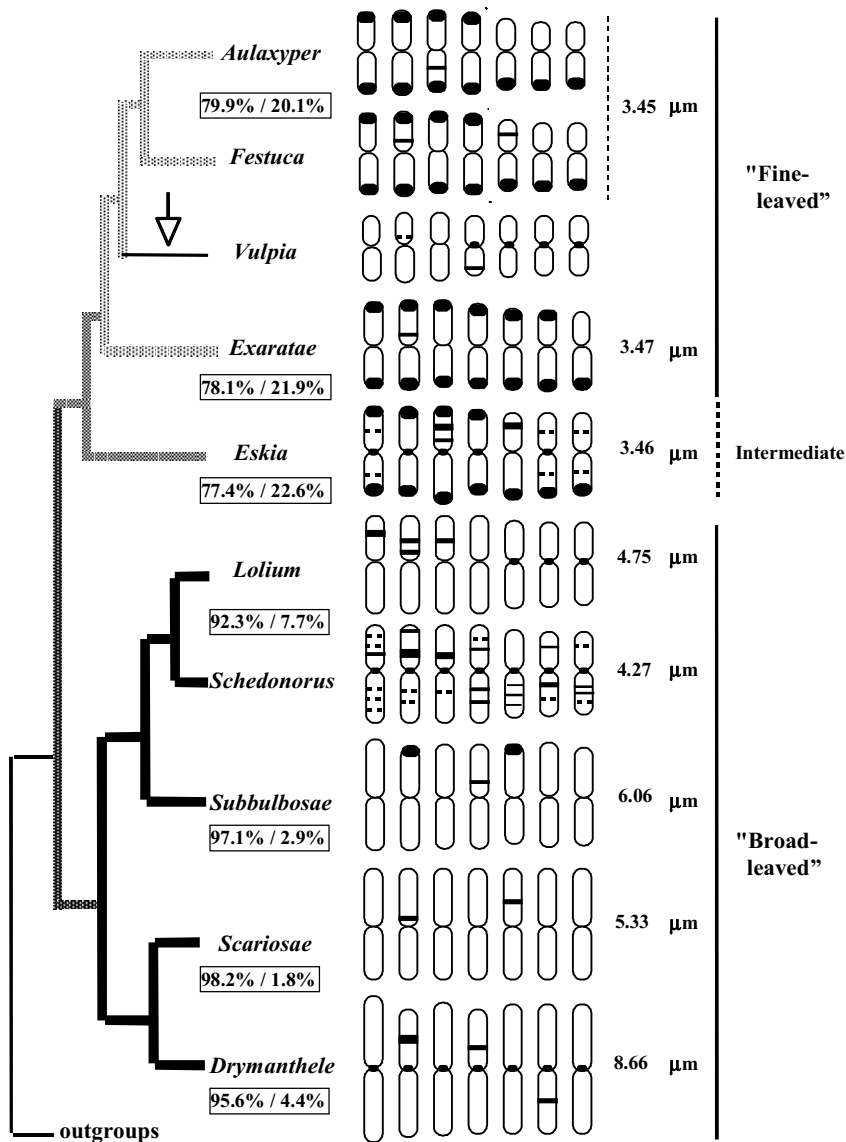


Fig. 3. Analysis of karyotype evolution in Loliinae. Variation in chromosome C-banding patterns, mean chromosome length (μm) and percentages of total euchromatin/total heterochromatin per karyotype is mapped for the main broad-leaved (*Festuca* subgenus *Drymanthele* (*F. drymeja*), section *Scariosae* (*F. scariosa*), section *Subbulbosae* (*F. paniculata*), subgenus *Schedonorus* (*F. pratensis*), plus *Lolium* (*Lolium* spp.)), intermediate (*Festuca* section *Eskia* (*F. pumila*), and fine-leaved (*Festuca* subsection *Exaratae* (*F. norica*), section *Festuca* (*F. ovina*), section *Aulaxyper* (*F. rubra*), plus *Vulpia* (*V. fasciculata*) Loliinae lineages. The arrow indicates a potential reversal to massive telomeric heterochromatin loss in *Vulpia*. Karyotype data was retrieved from Bailey & Stace [11], Dawe [46], and Thomas [160].

similar to those of the *Triticum* A-genome [133]. The most recently evolved fine-leaved *Festuca* and *Aulaxyper* lineages have the smallest submetacentric chromosomes and the highest contents in heterochromatin that is primarily located in the telomeric bands [11, 46] (Fig. 3), as those of the *Secale*-genome [46]. The mapping of structural karyotype characters onto the molecular topologies is in agreement with a scenario that predicts a strong reduction in chromosome size and a major increase in heterochromatin has occurred during the evolutionary history from more ancestral broad-leaved lineages towards the more recently evolved fine-leaved lineages of Loliinae (Figs. 1 and 3). A more detailed evaluation of karyotype evolution shows an intermediate C-banding pattern between those of the *Schedonorus* and *Lolium* taxa and those of the core fine-leaved *Festuca* and *Aulaxyper* taxa, which is present in the basal *Eskia* lineages of the fine-leaved clade, consisting of a mixture of intercalary and telomeric bands [46] (Fig. 3). The karyotype of the more advanced *Exaratae* taxa is similar to those of the more recently evolved *Aulaxyper* and *Festuca* species [46] (Fig. 3).

In contrast, the *Vulpia* karyotype shows small metacentric chromosomes with few heterochromatic bands that are mostly centromeric [11] (Fig. 3). This is in agreement with the low genome size reported for these plants [20]; however, it does not show any affinities with the karyotypic profiles of its closest sister, *Festuca* section *Aulaxyper* (Fig. 1). The most parsimonious interpretation for the remarkable distinctiveness of the *Vulpia* karyotype is that the strong reduction observed in its genome size can be correlated with a massive loss of telomeric heterochromatin coupled with a slight gain of centromeric bands. Perhaps this occurred during the acquisition of the annual habitat from a perennial chromatin-rich *Aulaxyper* ancestor.

Loliinae chromosome banding patterns appear to be lineage-specific to species-specific (Fig. 3), meeting the criteria for a discriminating systematic and evolutionary tool [46, 121]. Karyotype profiles have been proposed to reflect the breeding affinities of the intercrossable groups within the subtribe [46]. This is certainly accomplished in the intergeneric x *Festulolium* crosses where karyotypic patterns are similar in both *Schedonorus* and *Lolium* parents [46, 160] that share a common ancestry (Fig. 1). The *Drymanthele* + *Scariosae* lineages have had successful interfertile artificial crosses between the karyotype of *Drymanthele* vicariants, *Festuca drymeja* Mert. & W.D.J. Koch, and *E. donax* Lowe [22, 46], these being resolved as sister groups [77]. In

contrast, there are the less successful and sterile crosses between *F. drymeja*, *F. lasto* Boiss., and *F. altissima* All., which are more karyotypically diverse [22, 23, 46] and also more distant to one another on the phylogenetic trees [28, 77]. The genetic similarities of *Festuca scariosa* to species of *Drymanthele* as tested through artificial crosses [22, 23], is seen in their affinities in banding patterns [46], which in turn is supported by close phylogenetic placement [28, 77]. Despite these apparent coincidences, karyotype patterns are not always correlated with breedings affinities in Loliinae. Within the fine-leaved lineage, intergeneric x *Festulpia hubbardii* Stace & Cotton crosses are produced spontaneously though the *Vulpia* and *Aulaxyper* parents show strongly different C-banding profiles [11]. However, the *Vulpia* lineages are closely related to the red fescues [28, 164] (Fig. 1). Karyotype dissimilarities between *Aulaxyper* and *Vulpia* could be explained as a result of extensive heterochromatin loss in the annual *Vulpia* lineages, which probably has no effect on their ability to occasionally intercross [12].

Concordance between molecular phylogenetics and speciation processes along the festucoid lineages are also supported by cytogenetic data. Deeper insights into the genomic relatedness of several Loliinae lineages were obtained from FISH (fluorescence in situ hybridization) and GISH (genomic in situ hybridization) techniques [12, 63, 73]. The mapping of ribosomal DNA sites depicted decreasing degrees of relatedness within the *Drymanthele* lineage, from similar mapped *Festuca drymeja* and *F. donax*, to less similar *F. lasto*, and to the more distant mapping patterns of *F. altissima* [63]. This is in total agreement with the resolution of our molecular topology [77]. However, the speculations brought forward to favour either *Festuca scariosa* or *F. altissima* as the potential sources for the unknown *F. arundinacea* genome, and *F. scariosa* as the potential genome donor of *F. mairei* [63], first postulated from morphological and breeding affinities [22, 23], do not agree with the divergent phylogenetic placement found for these groups [28, 77] (Fig. 1). More exhaustive GISH procedures have confirmed the participation of the diploid *Festuca pratensis* and tetraploid *F. fenas* in the allohexaploid *F. arundinacea* and have demonstrated, despite initial indications [78], the non-relatedness of *Lolium* to this polyploid [73].

The impact of reticulation and polyploidy on the evolutionary processes of most angiosperms groups has been largely documented [135, 144, 152]. Recurrent introgression and polyploidization are common in many plant families and have been postulated as microevolutionary

scenarios for cladogenesis [134]. Grasses account for some of the highest percentages of polyploid taxa of hybrid origin [153]. Hybridization and polyploidy are commonly found in subtribe Loliinae where intergeneric hybrids between *Festuca* and *Lolium* (x *Festulolium*) and between *Festuca* and *Vulpia* (x *Festulpia*) occur spontaneously in the wild [3, 79, 97]. The extent of hybridization events among different *Festuca* groups and their closest allies (Fig. 4) can be used to trace past evolutionary processes obscured by biased dilution of parental traits in newly formed hybrid lineages [16, 80]. Spontaneous crosses between different festucoid lineages indicate the limits of their reproductive barriers [79, 97], whereas artificial crosses are a measure to assess the degree of genomic similarity [3, 17, 78, 80, 81, 82].

Within the broad-leaved clade, hybridization is broadly extended across the *Schedonorus* + *Micropyropsis* + *Lolium* lineage [78, 81, 82] though spontaneous crosses have been detected among closely related taxa in other broad-leaved groups, such as those involved in the origin of the *Subbulbosae* microspecies [95]. Success in artificial crosses was obtained among diploid members of *Festuca* subgenus *Drymanthele* that show wide geographic vicariance (*F. drymeja* and *F. donax*) and between *F. scariosa* (*Scariosae*) and taxa of subgenus *Drymanthele* [21, 22], thus confirming their close phylogenetic relationship [28, 77] (Fig. 1). The high ploidy levels manifested by representatives of *Leucopoa* p.p. (*F. kingii*, *F. spectabilis* Jan.) might also indicate a hybrid polyploid origin.

The most recently evolved *Schedonorus* + *Micropyropsis* + *Lolium* group shows a high number of spontaneous hybrids, especially those derived from intergeneric crosses between 'European' *Schedonorus* and *Lolium*. Natural hybrids between perennial and biennial species of *Lolium* (*L. perenne* and *L. multiflorum*) and three species of 'European' *Schedonorus* (*F. pratensis*, *F. arundinacea*, *F. lenas* (*F. gigantea*)) occur widely in sympatry in all six species combinations, though those hybrids are not completely sterile [80, 97]. However, all *Lolium* species can intercross with each other resulting in fertile offsprings [79, 80, 159]; as can the three previously mentioned representatives of 'European' *Schedonorus* [80]. Diploid *F. pratensis* and tetraploid *F. glaucescens* have been recognized as the genome donors of hexaploid *F. arundinacea* within the European clade based on data on the meiotic behaviour of F1 hybrids and derivatives [21, 32, 33, 101]. This was corroborated by RFLP probing [172] and genomic in situ hybridization [73]. Based on morphological similarities and chromosome pairing analyses of artificial

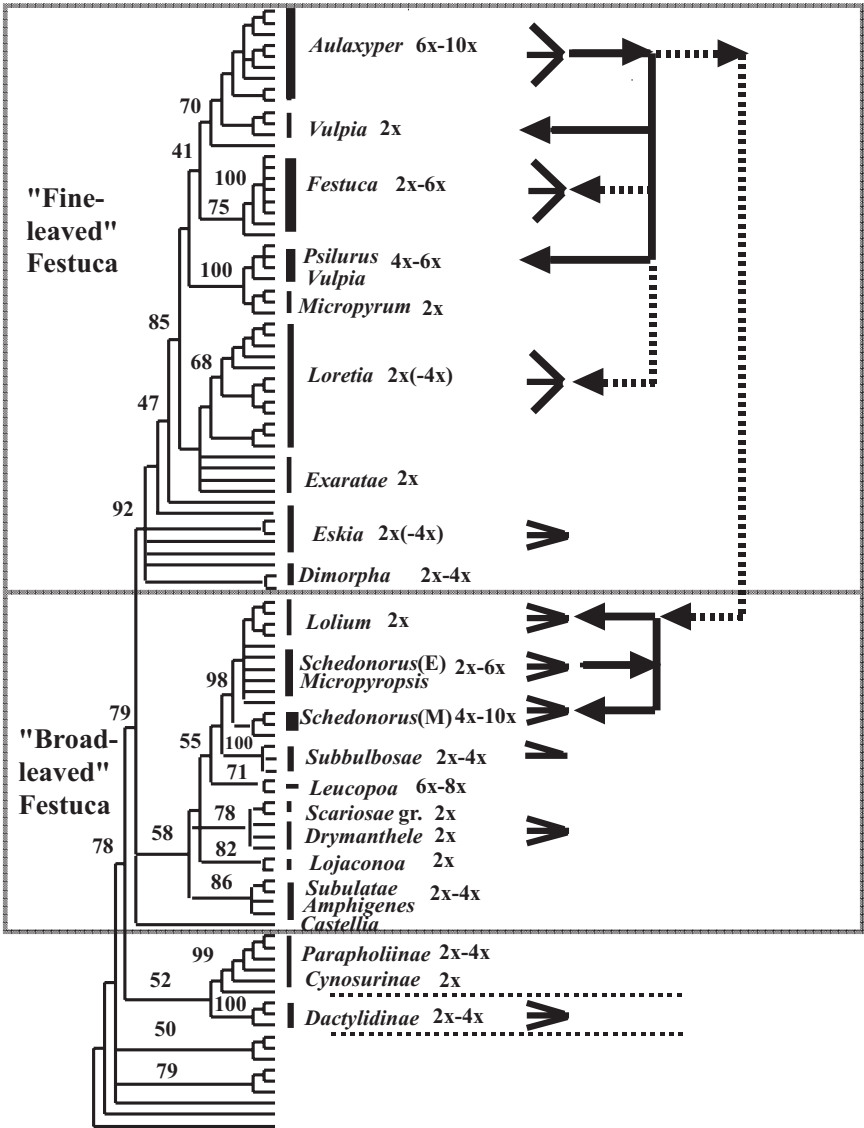


Fig. 4. Mapping of hybridization events of Loliinae taxa onto the combined nuclear and plastid DNA-most parsimonious tree (modified from Catalán et al. [28]).

hybrids, tetraploid *F. mairei* and *F. fenas* of the 'Maghrebian' clade have been postulated as putative parents of the highly polyploid taxa, *F. arundinacea* var. *atlantigena* and *F. arundinacea* var. *letourneuxiana* [34,



172]. However, the ultimate diploid ancestors of those lineages are either extinct or have not yet been identified. According to the present data and the phylogenetic resolution observed within the *Schedonorus* + *Micropyropsis* + *Lolium* group, it seems likely that a *F. pratensis*-type ancestor was involved in the origin of this diploid lineage and that *Lolium* stemmed out from it. Conversely, recurrent hybridization and amphipolyploidy probably gave rise to the separate polyploid-rich *Schedonorus* complexes of the 'European' and 'Maghrebian' subclades [76].

Extensive reticulation has also been detected within the fine-leaved clade where intergeneric crosses are common between representatives of *Festuca* section *Aulaxyper* and *Vulpia* [3]. Hybridization is relatively widespread within the perennial fine-leaved groups when the parental taxa are close enough, as manifested in the separate diploid to polyploid *Eskia*, *Festuca*, and *Aulaxyper* assemblages, though most of the resulting intra- and intersectional hybrids are usually highly sterile [15, 81, 82, 163, 164] (Fig. 4). By contrast, interspecific crosses are rare within the annual fine-leaved groups and artificial attempts among representatives of the annual *Vulpia* lineages were less successful resulting in sterile offsprings [15]. Higher rates of success were observed, however, in both spontaneous and artificial crosses between polyploid perennial and diploid to polyploid annual lineages, particularly those involving the red fescues. Representatives of the perennial hexaploid and octoploid *F. rubra* complex hybridize spontaneously with annual representatives of *Vulpia* sections *Vulpia* and *Monachne*, and artificially with those of *Vulpia* section *Loretia* in different ploidy combinations [3, 15, 145]. The *F. rubra* taxa could also hybridize with perennial species of *Festuca* section *Festuca* [15, 81]. Though most of these crosses produced sterile hybrids, backcross derivatives to the male *F. rubra* parent were fully fertile [3]. Increased genetic recruitment through recurrent introgression between representatives of *Festuca* section *Aulaxyper* and those of *Vulpia* and *Festuca* section *Festuca* has been interpreted as one of the major evolutionary trends within these recently evolving fine-leaved groups [17].

The crucial role played by the red fescues in the reticulation processes found within the Loliinae extends far beyond the fine-leaved group. Rare, intergeneric crosses between representatives of the *F. rubra* complex and *Lolium* have been documented either as spontaneous or as artificial derivatives [78, 80, 81, 113]. The *Aulaxyper* group is an important assemblage of species and might be one of the major

contributors to the speciation process in festucoids. Their ability to repeatedly backcross with diploid or low polyploid taxa from other lineages, acting as pollen donors and resulting in offsprings that mostly resemble these paternal parents, could explain the highly heterogeneous substitution rates observed within this lineage [30] and close phylogenetic placement near some annual (*Vulpia*, *Micropyrum*) and perennial (*Helleria*) lineages [28, 164]. Some of the highly polyploid taxa of this *Aulaxyper* complex are probably products of recent speciation fostered by introgression and polyploidization (Fig. 4).

Extended genomic introgression might explain the close evolutionary relationships observed between *Festuca* section *Aulaxyper* and representatives of *Vulpia* within the fine-leaved clade (Fig. 4). GISH analysis of natural and synthetic F1 x *Festulpia* hybrids demonstrated that telomeric heterochromatic regions of the *Festuca rubra* chromosomes are not present in the *Vulpia* genome, whereas euchromatic regions of the *Vulpia* genome hybridize to some extent to non-heterochromatic regions of the *Festuca* chromosomes [12]. This evidence further supports the proposed hypothesis of a potential loss of fine-leaved *Festuca*-specific repeated telomeric DNA sequences in *Vulpia* during the evolutionary trend towards reduction in genome size and acquisition of an annual life-cycle (Fig. 3). The occurrence of some heterogenetic pairing of *Festuca* and *Vulpia* chromosomes in meiotic bivalents of x *Festulpia hubbardii* plants [11, 12] supports their genetic proximity. However, the resolution of relationships between *Festuca* section *Aulaxyper* and the different *Vulpia* lineages (Fig. 1) is not satisfactorily explained, since representatives of several lineages of *Vulpia* sections *Vulpia* and *Monachne*, that spontaneously intercross with representatives of the *F. rubra* group, show different degrees of affiliation [28, 77, 164] (Fig. 1). These apparent incongruences might be caused by different recurrent intergeneric and interploidal introgressions of *Festuca* and *Vulpia* nuclear genomes, coupled with concurrent chloroplast captures that have obscured the derivation of clear phylogenetic hypotheses. Assortative matching and recurrent polyploid formation are common phenomena in several groups of angiosperms [134] and may be the likely hidden mechanisms behind the multiple origins of the diverging *Vulpia* lineages [164]. The similarities among the diploid representatives of *Vulpia* section *Vulpia* to *Festuca* section *Aulaxyper* indicate their common genomic ancestry, whereas the polyploid *Vulpia* lineages might have arisen at different evolutionary

times. Although the identity of their potential genome donors has not been established yet, the close genomic affinities of the red fescues with the polyploid *Vulpia* lineages via spontaneous intergeneric crosses and their morphological similarity to cosectional diploid taxa points towards the contribution of the *Aulaxyper* + diploid *Vulpia* lineage in their origin [164].

The secondary origin of polyploidy in *Festuca* and close allies can also be deduced from our phylogenetic trees. For those lineages that include diploid and polyploid taxa, i.e. *Schedonorus* + *Micropyropsis* + *Lolium* clade within the broad-leaved lineage and the *Aulaxyper* lineage within the fine-leaved clade, diploid species always align basally (Fig 1). The nature of polyploidy across *Festuca* has long been debated [78]. The regularity in size, symmetry and banding patterns of individual karyotypes [46] and the high percentages of regular meiotic pairings [113] have been interpreted as evidence of autopolyploidy for some *Festuca* lineages, i.e. *Festuca* sections *Festuca* and *Aulaxyper*. However, in other cases, the slight asymmetry of karyotypic profiles and the ratio between homologous vs. homeologous and heterologous pairing in artificial interspecific hybrids have been postulated as evidence of segmental polyploidy (*Festuca* sections *Festuca* and *Subbulbosae*) [46, 100] or true allopolyploidy (*Festuca* subgenus *Schedonorus*) [32, 33, 101]. This last mechanism was corroborated by the explanation of the origin of hexaploid *Festuca arundinacea* from diploid *F. pratensis* and tetraploid *F. fenas* through different cytogenetic analyses [63, 73, 172]. Based on meiotic behaviour of artificial hybrids, this mechanism has also been proposed for the derivation of the polyploid taxa of the *Schedonorus* lineage [33] and the *Aulaxyper* lineage [78]. Ribosomal DNA mapping has also confirmed that taxa previously considered to be autopolyploids, such as the tetraploid *F. apennina* De Not., are of hybrid origin with one genome from diploid *F. pratensis* and another genome of unknown origin (J.A. Harper, personal communication).

The regulatory mechanism of chromosome pairing in *Festuca* seems to be under genetic control in the allopolyploids [78] and apparently evolved in parallel along the broad and fine-leaved festucoid lineages (Fig. 4). Based on evidence compiled from chromosome pairing of intergeneric *F. arundinacea* x *Lolium multiflorum* Lam. and *F. rubra* x *Lolium perenne* hybrids and derived amphidiploids, Jauhar [78] inferred that the diploid-like meiosis of the hexaploid *F. arundinacea* and *F. rubra* species is regulated by a gene system that represses homeologous pairing

in disomic dosage, but is ineffective in hemizygous dosage, similar to the genetic-controls observed in hexaploid wheats and oats. The extent of this mechanism across the festucoids was indirectly confirmed by the heterologous pairing observed in the wild and artificial x *Festulopia hubbardii* hybrids [11, 12]. The analysis of intraspecific *F. arundinacea* crosses from different geographical ecotypes allowed Jauhar [78] to discern the presence of this regulatory system acting in the diallelic homozygous fertile crosses from its absence in the monoallelic hemizygous sterile ones. The extension of this regulatory system in subtribe Loliinae probably favoured the stabilization of allopolyploids originating from closely related parental species in different broad-leaved and fine-leaved lineages. Breakdown of this genetic control caused by occasional intergeneric or interspecific hybridizations could have given rise to semi-fertile hybrids which may have represented windows of opportunity for further speciation via introgression or amphidiploidization in the Loliinae.

## EVOLUTIONARY RATES ACROSS THE FESTUCOIDS

Bayesian phylogenetic analyses allowed inferences on the differences of evolutionary rates across the Loliinae lineages based on differences in their respective branch lengths [28, 164]. Grasses have shown to be one of the most rapidly evolving lineages within monocotyledons and to support in most cases, though not always, to the Minimum Generation Time (MGT) hypothesis. This predicts that annual or short-lived perennials, which propagate rapidly, show higher mutational rates than their respective long-lived perennial congeners, indicating a release from stabilized selection [53, 54]. Different evolutionary trends in rate heterogeneity have also been associated with the Speciation Rate (SR) hypothesis, which assumes that higher cladogenetic events are associated with higher substitutional rates [18, 19]. A Reticulation-Polyploidization (RP) hypothesis to explain the higher fitness and adaptive success shown by polyploid festucoids with respect to their close diploid relatives in connection with their differential mutation rates was proposed by Catalán et al. [30].

Relative rate tests [119, 120] performed between the main lineages of Loliinae and its close allies for the ITS and plastid *trnL*F genome regions made it possible to test those evolutionary hypotheses [30, 164]. A strong correlation was found between substitution rates in the nuclear

and plastid genome regions and the life-cycle strategies shown by these groups [30, 164]. Evolutionary rates within the Loliinae vary enormously showing a general trend from slowly-evolving perennial lineages towards rapidly-evolving annual ones. The broad-leaved groups evolve in general terms at a lower pace than the fine-leaved ones. The broad-leaved perennial assemblages of *Leucopoa*, *Drymanthele*, and *Subbulbosae*, which include some of the tallest and most robust representatives of *Festuca*, showed the lowest rates of substitutions, followed by those of *Scariosae*, *Pseudoscariosa*, and *Lojaconoa*, and then by those of the more rapidly evolving *Schedonorus* + *Micropyropsis* + *Lolium* lineage. Within the last group, the European *Schedonorus* evolves at a lower pace, followed by *Micropyropsis* and the Maghrebian *Schedonorus* group, and then by *Lolium*, with the annual *Lolium* taxa showing the most accelerated substitution rates of all the broad-leaved groups and as fast as (or faster than) some of the fine-leaved groups [30].

Groups of intermediate placement in our phylogenetic trees (*Dimorpha*, *Leucopoa* p.p.) also show intermediate rates of mutation between the slowly-evolving broad-leaved groups and the fast-evolving fine-leaved groups. The more advanced fine-leaved *Festuca* groups show a trend towards higher substitutional rates in both nuclear and plastid sequences. Rate heterogeneity ranges are lowest in the old relict perennial groups (*Eskia*, *Exaratae*), intermediate in the more recently evolved perennials (*Festuca*, *Helleria*, *Aulaxyper*), and fastest in the newly derived annual lineages. Within the last group, different assemblages of the genus *Vulpia* and its close allies show significant differences from most of the remaining fine-leaved groups [30, 164].

Annual lineages evolve significantly faster than the perennial ones within both Loliinae groups, thus supporting the MGT hypothesis [30]. MGT mechanisms are operating in the rapidly evolving ephemeral groups of these grass lineages even if the biological factors that regulate these processes have not yet been deciphered [54, 112]. The changes in the evolutionary rates between slow-evolving perennials and fast-evolving annuals are interpreted as the probable consequence of a release from stabilized selection followed by the ephemeral groups that allowed the acquisition of rapid adaptive changes to new environmental habitats [57]. Our preliminary analyses also favour the speciation rate hypothesis within Loliinae, as indicated by the higher diversifying rates shown by the higher accelerated fine-leaved Loliinae lineages compared to the lower-

diversification rates of the slower mutational broad-leaved lineages [30]. Interpretation of the wide array of monotypic and small-sized genera described within the fine-leaved *Festuca* clade might be a direct consequence of the higher speciation rates developed by these ephemeral groups [164].

Increased evolutionary rates are correlated with increased levels of ploidy [30], supporting the reticulation/polyploidization hypothesis for some festucoid lineages. This evolutionary scenario has developed independently along the two main clades of Loliinae, as exemplified by the *Aulaxyper* and the Maghrebian *Schedonorus* groups within the fine and broad-leaved *Festuca* lineages, respectively. These two groups encompass highly polyploid taxa (8x-10x) that are presumably derived from their respective lower-ploidy-level relatives. Diploid perennial lineages display the lowest mutational rates in both broad and fine-leaved *Festuca* lineages and the broad-leaved ones evolve significantly more slowly. Conversely, diploid annual taxa tend to show higher mutational rates than the ephemeral polyploids [30]. Although hybridization may have equally affected diploid and polyploid lineages, it is more common within the latter groups; this circumvented the new sterility barriers via recurrent introgression and polyploidization [3, 79, 144, 153]. Polyploidization is expected to increase the rate of variability of the nuclear genome concordantly with the accumulation of more gene copies [135] but the plastid genome should have lower levels of variability. However, concurrent rates of nucleotide substitutions in the two genomes, detected for both diploid and polyploid lineages of the Loliinae, may indicate other concerted nuclear and cytoplasm replication mechanisms in these plant cells [54].

High mutation rates may have negative consequences on phylogenetic reconstructions due to the loss of deep phylogenetic signal to give undesirable results such as long-branching attraction and site saturation effects [68, 170]. The lack of resolution observed at some subbasal nodes of our festucoid tree (Fig. 1) and some unexpected relationships could be associated with disturbance caused by increased levels of homoplasy displayed by the most rapidly evolving groups [30]. In *Lolium* the annual taxa show high accelerated mutational rates significantly different from most of the slowly-evolving perennial lineages. The high levels of morphological variability detected in *L. rigidum* and *L. canariense* Steud., which moved some authors to describe different infraspecific and specific taxa out of those complexes [130, 159], are thus

correlated with their higher substitution rates. Within *Vulpia*, homoplasy may be enhanced in these highly evolving lineages altering the phylogenetic inference. However, the concurrent reconstruction of a consistent *Loretia* + *Monachne* + *Spirachne* + *Apalochloa* + *F. plicata* + *Ctenopsis* clade supports a common ancestor for all these *Vulpia* lineages (the ‘*Loretia* assemblage’) except for typical section *Vulpia* [30, 164].

The switch from perennial towards annual life-cycle probably represents a more ancestral evolutionary phenomenon, experienced independently in the two main festucoid lineages as manifested by the mainly diploid nature of the ephemeral lineages (Fig. 4). This scenario could be concurrent with strong genome rearrangements caused by several heterochromatin losses, deduced from cytogenetic analyses [11, 12]. Reticulate processes involving recurrent hybridization and polyploidy probably constitute secondary evolutionary events that have affected some of the most recently evolved perennial lineages of broad and fine-leaved *Festuca* and a few annual fine-leaved lineages. Both polyploids and their diploid parents show similar karyotypic and ribosomal DNA mapping profiles [46, 161], which indicate less evident genome rearrangements. Although acceleration rates are significantly different across the annual lineages, the polyploid complexes show relatively higher rates, indicating that reticulation perhaps fostered the substitutional rates of these groups through the addition of new gene-pools [30]. Both scenarios agree, partially, with the speciation-rate hypothesis, as annuals and highly polyploid taxa show a wider array of taxa within each lineage than their congeneric or cosectional relatives. However, the annual lineages show distinctive traits that were used to classify them as different genera [39, 169], and the polyploid complexes are formed by a series of microtaxa that can hardly be morphologically differentiated from each other [102]. These lines of evidence add support for an older divergence of the annual lineages from their respective common ancestors and for a secondary and more recent divergence of the polyploid lineages.

## BIOGEOGRAPHICAL AND PHYLOGEOGRAPHICAL PATTERNS OF FESTUCOID LINEAGES

### Biogeography

Molecular studies provide the basis for discerning the biogeographical patterns shown by different Loliinae lineages [13, 14, 28, 52], confirming



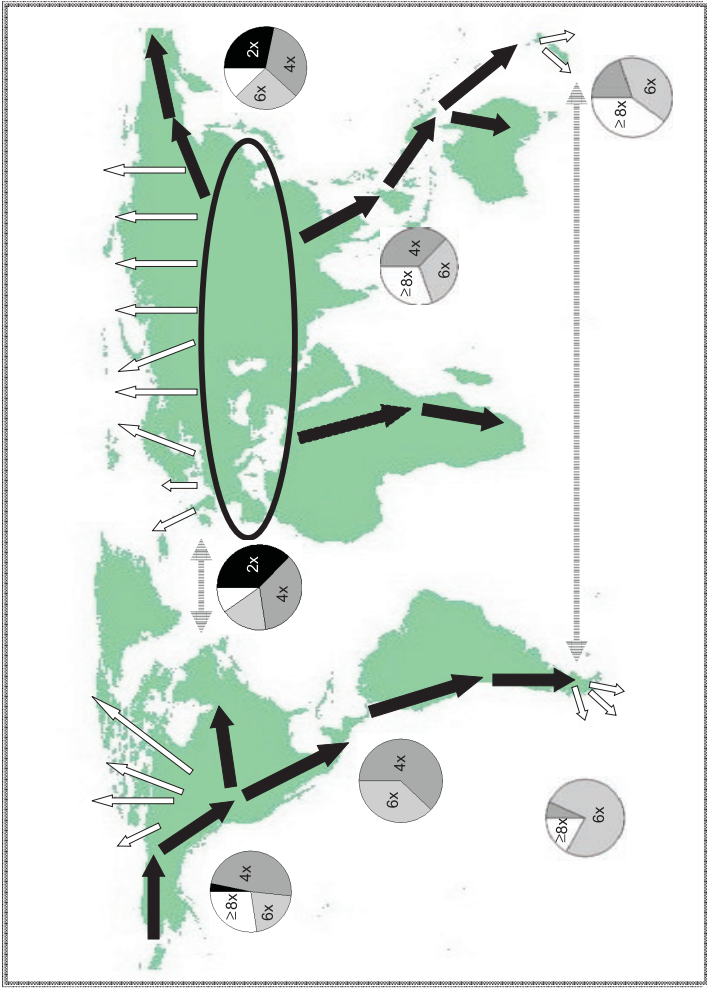
previous hypotheses based on geographical distribution of both regional endemics [64] and chromosome races [48]. Most recent phylogenetic surveys [28, 77] allow us to distinguish primary and secondary radiation centres located in different areas of the Old and New World, and to postulate evolutionary scenarios for the expansion of the festucoid lineages from the northern hemisphere towards the southern hemisphere (Fig. 5).

The Mediterranean–Asian region was the likely primary centre of speciation for both broad and fine-leaved lineages, since most diploid Loliinae representatives are endemic to this region and align basally in their respective clades [28]. Diploid ephemeral taxa (*Lolium*, *Vulpia*, *Narduroides*, *Wangenheimia*, *Ctenopsis*, *Micropyrum*) are native to the pan-Mediterranean–Asian area, whereas old relict diploid perennial lineages (*Subulbosae*, *Drymanthele*, *Scariosae*, and *Pseudoscariosa* within the broad-leaved clade, and *Eskia* and *Exaratae* within the fine-leaved clade) are distributed in the Mediterranean region and, to some extent, in Eurasia [28]. Karyological reports indicate that almost all the American and southern hemisphere fescues studied are polyploid [41, 48]. Diploid taxa predominate in the circum-Mediterranean region and across the main Asian mountain ranges; tetraploids are prevalent in northern and Central America; hexaploids in Africa; and octoploids with hexaploids constitute the main contingents in the austral regions of Patagonia [48] and New Zealand [41] (Fig. 5). Dubcovsky & Martinez [48] highlighted the noticeable absence of diploid species of *Festuca* in the southern hemisphere.

A clear north-to-south cline of increasing ploidy levels can be envisaged from the pan-Mediterranean–Asian belt towards America and the African/Austropacific continents (Fig. 5). This cline is paralleled by a similar south-to-north increase in ploidy levels from the temperate pan-Mediterranean–Asian regions towards the arctic zone where tetraploids, hexaploids and higher polyploids predominate over diploids [48]. However, the different northern and southern hemisphere polyploid lineages probably had different evolutionary origins.

Combined molecular phylogenies of Loliinae recover the sequential divergences of polyploid American and New Zealand *Festuca* clades that are nested in an intermediate position between the broad and fine-leaved lineages or within the last clade [77] (Fig. 1). All the Holarctic polyploid lineages are derived from clades where the basal diploid taxa are of pan-





**Fig. 5.** Biogeographical hypothesis of primary and secondary radiation centres of *Festuca* and related genera, based on phylogenetic inference and on chromosomal geographical distribution. Eurasia is postulated as the likely primary centre of divergence of the diploid festucoids in the late Tertiary. Potential colonization pathways towards America, Africa, and the Austropacific region are indicated by black arrows. Secondary speciation centres of polyploid lineages related to the Quaternary climatic changes are indicated by grey arrows. Occasional long distance dispersal events in the perianctic and the Holarctic areas are indicated by grey arrows. Circles indicate the relative percentages of ploidy levels in each continental area (modified from Dubcovsky & Martínez [48]).

Mediterranean–Asian origin (Fig. 1). Thus, it could be speculated that the Mediterranean and European-Asian region (Eurasia) was the centre of origin for the old relict *Festuca* elements and that different migration routes allowed the more aggressive polyploid lineages to colonize other continents where successive radiations increased the spectrum of the extant taxa (Fig. 5). This scenario is likely to have predated the Pliocene and Pleistocene glaciations and could be associated with late Tertiary transcontinental land bridges that connected the Asian and American continents through the Beringian passage and the North American and South American masses after the closure of the Caribbean pass [77]. According to the present phylogenetic evidence (Fig. 1), colonization of the American continent probably took place on at least six separate occasions and that of the Neozeylandic region on at least two occasions, each followed by consecutive secondary radiations [77]. This hypothesis is concordant with molecular clock estimates based on nucleotide substitution rates of the festucoid plastid genome that estimate nine mya (million years ago) for the divergence of the broad and fine-leaved lineages [35], indicating a late Miocene origin for the basal lineages of *Festuca*, and two mya for the divergence of the *Schedonorus* + *Lolium* lineage [35, 52], indicating a Quaternary origin for this more recently evolved group.

Similar ancestral biogeographical north-to-south colonization and radiation scenarios have been postulated for the widely distributed grass genera *Poa* [136] and *Brachypodium* [129]. This hypothesis could be extended to other pooid groups where diploids predominate in the northern hemisphere, but are absent south of the equator [48, 64, 98]. The nesting of polyploid South American and New Zealand lineages within both the broad and fine-leaved lineages (Fig. 1) is better interpreted as a result of distinct colonization and secondary divergence episodes rather than of recent repeated long distance dispersal events. However, one of these lineages supports a floristic link between the New World and New Zealand [77], as evidenced by the sister-group relationships between the Neozeylandic clade I and the western North American *Festuca californica* Vasey (Fig. 1). Although the present taxon sampling of *Festuca* s. l. does not allow further speculation on this hypothesis, a major radiation of New Zealand taxa from colonizers that dispersed from North America to South America, and then to the Neozeylandic region rather than from Asia, has been proposed for the bluegrasses (*Poa*), based on a biogeographical analysis of plastid

restriction site data [136]. Several Asian-western American and amphitropical disjunctions observed in some Loliinae clades, such as those of the broad-leaved Asian-American clade I (Fig. 1), suggest that the number of dispersal events between the Old World to North America and then to South America might be more numerous than what is detected with the present data [77].

The more recent secondary radiation associated with the oscillatory climatic changes of the Quaternary probably fostered the speciation of the Holarctic polyploid lineages and some of the southern hemisphere groups (Fig. 5). Clear examples of the former case can be found within the Mediterranean and Eurasian broad-leaved *Schedonorus* and fine-leaved *Aulaxyper* lineages, which show a broad array of ploidy levels ranging from diploids to decaploids (Fig. 1). Several documented cases support the hypothesis that polyploid plants were more successful than diploids in colonizing deglaciated areas [24, 154, 155], and that recurrent polyploid formation spanned over different scales in time and space during the Quaternary from low-level to high-level polyploid taxa [1, 24]. Successive cycles of contraction and expansion of populations concomitant with the advances and retreats of the ice cover ultimately led to repeated contacts between previously isolated divergent lineages resulting in increasingly intricate polyploid complexes through hybridization and polyploidy [1, 24, 65, 66]. In *Schedonorus* and *Aulaxyper* lineages, putatively old and scarce diploid species are outnumbered by different series of allopolyploid taxa, now widespread in the previously glaciated areas of central and northern Europe and the highest Mediterranean mountain peaks. The highly heterozygous nature of allopolyploids can help buffer against inbreeding and genetic drift. Polyploidization has been hypothesized as the main evolutionary force that favoured the survival of the newly arisen taxa during the coldest periods of the glaciations, as well as their subsequent success in colonizing the deserted areas across a wide range of ecological habitats [24, 152, 153].

The high polyploid complexes found in the subantarctic peripheral area that was exposed to dramatic changes during Quaternary glaciations might mirror the pattern observed in the Holarcticone. Secondary cladogenic events possibly resulted in the diversification of closely related lineages of *Festuca*, such as the hexaploid South American clade II and the hexaploid Neozeylandic clade II [77] (Fig. 1). Some circum-antarctic taxa share with other circum-arctic congeners their ability to reproduce

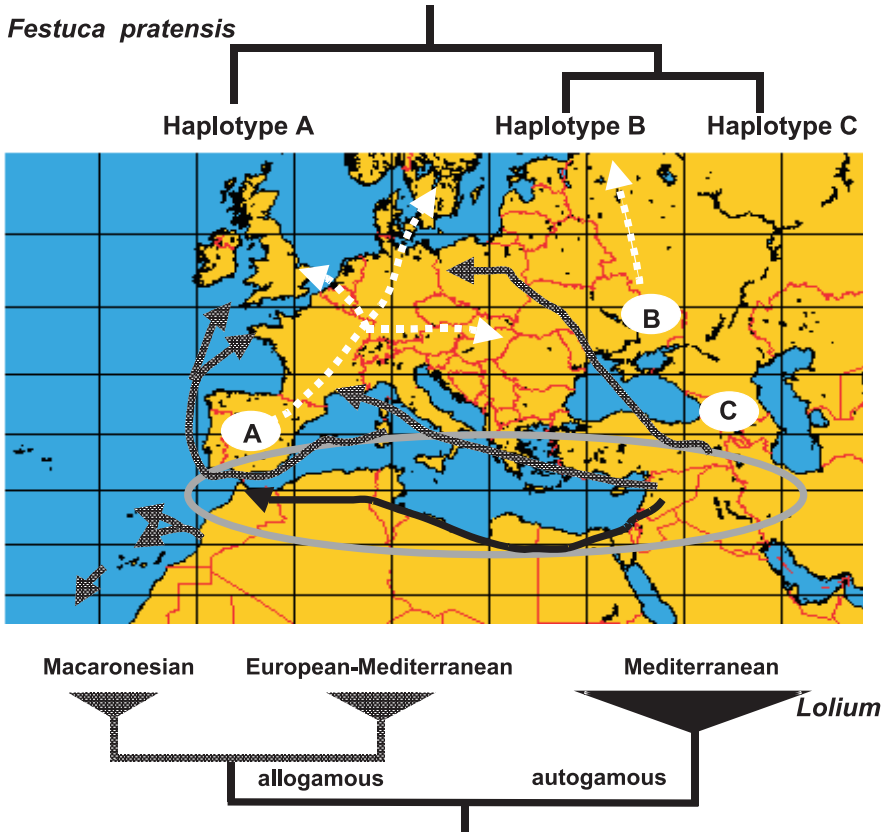
vegetatively, a common character in polar plants, and of inbreeding, an uncommon derived trait in *Festuca* [41, 51]. These traits probably facilitated the colonization and survival of festucoids in the severe climates.

## Phylogeography

Using ITS phylogenetic inference [76], plastid DNA molecular clock estimates [35, 52], and pollen to seed flow ratios deduced from plastid RFLP and allozyme data [13, 14], more detailed phylogeographic scenarios have been postulated for the origin and dispersal of the *Lolium* lineages and *Festuca pratensis* (Fig. 6). Charmet et al. [35] and Fjellheim et al. [52] concur that early Pliocene divergence of the *Festuca pratensis* and *Lolium* lineages from their common ancestor occurred approximately two mya. However, they differ in the proposed postglacial colonization patterns followed by each of these lineages that were probably connected with the expansion of agriculture during the Holocene in the European-Mediterranean area [14, 52].

The plastid data revealed that both the native races of meadow fescues (*F. pratensis*) and eight species of *Lolium* probably diversified at the beginning of the Pleistocene (one mya) but experienced strong bottlenecks during the most recent glaciations (Riss and Würm, < 150,000 ya), resulting in the extermination of most European populations, except those sheltered in the circum-Mediterranean glacial refugia [35, 52]. The phylogeographic patterns deduced for *F. pratensis* suggest that the glacial survivors were isolated in three southern European refugia located in the Iberian Peninsula, in southeastern Europe and in western Caucasus, and that the contemporary variation detected in *F. pratensis* could be explained by northwards expansion of the Iberian haplotype along western Atlantic Europe and the southeastern haplotype migration towards central and northern Europe, coupled with a lack of expansion of the Caucasian haplotype (Fig. 6). This migration scenario is concordant with that postulated for other higher plants that were sheltered in the three main southern European Mediterranean refugia [40, 158].

Molecular evidence was used to postulate a distinct origin and migration scenario for the *Lolium* species implying human intervention during the spread of agriculture from the Fertile Crescent towards Europe and the Mediterranean basin after the last glaciation [14]. However, such



**Fig. 6.** Phylogeographic patterns of *Festuca pratensis* and *Lolium* in their native pan-Mediterranean and European areas. *F. pratensis*: Postglacial expansion of western and eastern European plastid haplotypes from their respective Iberian and eastern European glacial refugia, and non-expansion of the Caucasian haplotype (white circles and arrows) (taken from Fjellheim et al. [52]). *Lolium*: Potential ribotype distribution area of an ancestral Mediterranean *Lolium* lineage (grey circle) that diverged into present Mediterranean autogamous and allogamous taxa, and further split and colonization pattern of a Macaronesian allogamous lineage (thin dotted arrows) (taken from Inda et al. [77] and unpublished data). Hypotheses on recent Holocene-Neocene expansions of ryegrass plastid haplotypes and allozymic profiles associated with the spread of the agriculture from the Middle East to the western Mediterranean area and to Europe (*L. rigidum*: North African migratory route (black arrows); *L. perenne*: South European and Central European migratory routes (thick dotted arrows) (taken from Balfourier et al. [14])).

a recent Middle East origin and Neocene dispersal of the *Lolium* taxa are in disagreement with nuclear ITS phylogenies and with geographical data that indicate *Lolium* lineages probably diverged and expanded before the Holocene [76] (Fig. 6). The origin of the *Lolium* taxa has been controversial as different hypotheses have been proposed for the alternative earlier splits of perennial vs. annual species and of outbreeding vs. inbreeding species [14, 35, 50, 100]. Classical hypotheses regarded the perennial/biennial outbreeders (i.e. *Lolium perenne*, *L. multiflorum*) as the most primitive species of the genus, that through progressive reduction gave rise to the annual outbreeders (*L. rigidum*) and then to the annual inbreeders (i.e. *L. temulentum* L., *L. remotum* Schrank, *L. persicum* Boiss. & Hohen.) [50]. However, karyotype analysis indicated that the inbreeding species had 40% more DNA than the outbreeding species, and that *Lolium perenne* features a derived lineage characterized by the shortest chromosomes in *Lolium* with the lowest amounts of heterochromatin [100, 160]. The apparent earlier divergence of the self-fertilizing *Lolium* species, with respect to the more recent origin of the outbreeding species, has also been supported by RAPD [146] and plastid [35] data, though this evolutionary trend does not agree with the one proposed for the fine-leaved *Festuca* lineages and *Vulpia* [43, 46, 164].

Despite previous proposals on the evolution of *Lolium*, exhaustive molecular phylogenetic studies of the *Schedonorus* + *Micropyropsis* + *Lolium* group recovered a clear ribotypic reproductive and geographical structure for the ryegrasses indicating the earlier split of the two autogamous vs. allogamous Mediterranean lineages followed by a further branching off of an allogamous Macaronesian clade (*Lolium canariense*, *L. edwardii* H. Scholz, Stierst. & Gaisb.) [76] (Fig. 6). It has been also demonstrated that the annual lineages (i.e. *L. canariense*, *L. rigidum*) mutate faster than the perennial lineages (*L. perenne*), arguing for a more rapid adaptive trend of the ephemeral lineages irrespective of their divergence times [30].

Based on the distribution of *Lolium perenne* and *L. rigidum* plastid haplotypes in the European and pan-Mediterranean areas and on calculations of pollen/seed flow ratio, Balfourier et al. [13, 14] concluded that the low values for these wind-pollinated ryegrass species apparently favoured a seed dispersal scenario, which was estimated to be concomitant with the expansion of the agriculture in the Neocene.

Three different eastern to western man-mediated migratory clines were hypothesized for the dispersal of the *Lolium rigidum* (north-African pathway) and the *L. perenne* (south-European and central-European pathways) plastid haplotypes [14] (Fig. 6). This Holocene-Neocene scenario of a historical single origin of all *Lolium* species from an inbreeding ancestor in the Fertile Crescent and subsequent expansion towards the west in conjunction with agriculture, disagrees with the present geographic distribution of native *Lolium* species [159] and with topologies inferred from nuclear data [76] (Fig. 6). Despite the monophyletic origin recovered for the *Lolium* lineage (Fig. 1), the divergence of the allogamous Macaronesian endemic clade (*L. canariense*, *L. edwardii*) (Fig. 6) probably predated the expansion of agriculture in the Old World. The Macaronesian *Lolium canariense* and the El Hierro island endemic *L. edwardii* probably represent old splits of an annual outbreeding *Lolium* lineage that colonized the Canary, Madeira, and Cap Verde isles from the near western European-African continent, rather than from the more distant eastern Mediterranean or Middle Eastern areas, as documented for most of the Macaronesian endemic angiosperms [26]. The presence of native *Lolium canariense* in the Macaronesian isles that were not populated before the 15th century (Madeira) clearly refutes the hypothesis of man-mediated transport of all *Lolium* species in Holocene-Neocene times and supports an earlier preglacial or interglacial expansion scenario for the *Lolium* lineages along the Mediterranean basin [76]. However, very recent eastern to western dispersal routes fostered by early Neocene farmers might have occurred for some native continental species that could be spread as crop weeds (*L. rigidum*) or as animal fodder (*L. perenne*) [13, 14].

## CONCLUSION

Our most recent and exhaustive phylogenetic surveys of the festucoids have covered a broad range of subgeneric and sectional ranks of *Festuca* and its close allies, making it possible to build a comprehensive framework to interpret a classification of the evolutionary history of subtribe Loliinae [29, 77]. The extended phylogenetic analyses have developed concomitantly with other crucial sources of data on genome structure, hybridization and polyploidization, which together have brought forward new hypotheses on macroevolutionary and microevolutionary speciation events for different lineages and taxa of this



important group of grasses. Ongoing research, however, focuses on the analysis of other genome sources, such as nuclear single copy genes, mitochondrial and plastid genes, which, similar to other grass tribes [85, 86, 87, 106, 107, 114], could facilitate a better understanding of the evolutionary mechanisms implied in the divergence of both deep and terminal branches of the festucoids.

Detailed analyses of genomic interaction between different gene copies, genome complements and cytoplasmic genes [104] could be decisive for the unravelling of the origins of the polyploids in this festucoid group where allopolyploidy seems to have been the driving evolutionary force [79]. Further research on gene duplication, gene expression, epistasis and pleiotropy could shed light onto the phenotypic and adaptive differences observed in some Loliinae species, as indicated for various Poaceae taxa [85, 87, 88]. Developmental genetics, investigated through genome colinearity and functional genome analysis, is a promising field of research explored in several grass groups of economic importance [86, 87], including Loliinae [10, 83]. It has provided new insights into the qualitative and quantitative morphological differences observed among taxa as results of different models of gene mutations and gene expression-repression mechanisms in different organs and life-stages [88, 89]. Comparative genome mapping detected highly orthologous and colinear *Lolium perenne* and *Festuca pratensis* linkage groups that also showed a syntenic relationship to the homeologous Triticeae, rice and oat genomes [10, 83]. Similarities in karyotype C-banding patterns, regulatory chromosome pairing genes and major nuclear genome arrangement support the hypothesis of the grasses (or at least the most recently evolved Pooideae) as a single functional genetic unit. The genetic basis for the heterochronic to heterotopic phenetic changes detected in a few model grass plants analyzed so far [84, 86] extends beyond those cases and is present in the Loliinae [74, 110].

Another interesting, though yet unexplored, field of evolutionary research is that of coevolution between some Loliinae lineages and their endosymbiotic fungi [109, 123, 124, 128, 156]. Endophytes of genus *Epichloë* range in a continuum from vertically transmitted seedborne asexual mutualists (called *Neotyphodium*) to horizontally transmitted sexual obligated pathogenic antagonists [128]. The horizontally transmitted species have haploid genomes and speciate cladistically (bifurcate branching), whereas the strictly seedborne mutualists are



interspecific hybrids (reticulate) with selective advantage for the symbiotic habit [109]. Despite the cross-specific and cross-tribal jumps of sexual *Epichloë* species, they only express their sexual state, when symbiotic with a particular host genus or tribe [127]. Different authors have suggested that the strong mutualistic stamp of endophytes and their hosts could be largely historical and system-based [38, 123]. Some evidences presented by Moon et al. [109] indicated that there might be some history of association between asexual *Neotyphodium* taxa and *Schedonorus* + *Lolium* taxa (*F. arundinacea*, *Lolium rigidum*, *Lolium* spp.), as four hybrid endophytes showed *tub2* and *tef1* alleles that joined in a well-supported ‘*Lolium*-associated’ clade.

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# **PLANT GENOME: BIODIVERSITY AND EVOLUTION**

Volume 1, Part D

**Phanerogams (Gymnosperm) and  
(Angiosperm-Monocotyledons)**

# Molecular Genetic and Cytogenetic Evidences Supporting the Genome Relationships of the Genus *Avena*

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## ABSTRACT

The genus *Avena* is an ideal system for investigating genomic organization and co-evolution of different genomes in nuclei to which they are common. This genus includes species with different degrees of ploidy and diverse genome composition. Cytological and cytogenetic studies based on chromosome number, morphology and pairing behaviour in hybrids, have been used to explain the evolutionary pathway of its polyploid species. Recently, molecular genetic and cytogenetic approaches, including the analysis of DNA sequences, have been used to disclose genome relationships within the genus. In the present study, fluorescence in situ hybridization using ribosomal sequences and two satellite DNA sequences, As120a (specific to the A-genome chromosomes) and Am1 (specific to the C-genome chromosomes), were used to identify the genome composition of a new tetraploid species. The results shed new light on the putative ancestors of the polyploid species. This work also reviews the present understanding of the evolution of the genus *Avena*, emphasizing the differences among its species and their genomes, as shown by molecular genetic and cytogenetic techniques.

**Key Words:** *Avena*, molecular cytogenetic, molecular analysis, *A. insularis*

## INTRODUCTION

Understanding the genome relationships between and within plant species is very useful to cytogeneticists, plant breeders, evolutionists and molecular biologists. Since the publication of the monograph *Cytogenetics of Oats* [65], continued efforts have been devoted to understanding the genome composition of oat species (diploid, tetraploid and hexaploid species). The common cultivated oat (*Avena sativa* L.) is the world's sixth most important cereal crop, grown in many areas where climatic conditions are unfavorable to the production of major cereals [55, 69]. Oats is a major crop for three main reasons: (1) it shows broad adaptation, (2) it can provide both human food and animal fodder, and (3) it has a higher concentration of well-balanced proteins than other grains [59].

Attempts have been made to establish the genome relationships between the diploid and polyploid species. Genome differentiation was initially based on descriptions of karyotypes and the results of cytological studies on interspecific hybrids [65]. Modern technology has, however, provided a more comprehensive image of these genome relationships. For example, close and distant relationships between specific genomes have been revealed by genomic in situ hybridization (GISH) [7, 25,45] and fluorescence in situ hybridization (FISH) with molecular probes [13, 21, 31, 51]. In addition, the relationships among *Avena* species with different ploidy levels have been identified through the use of molecular markers, such as restriction fragment length polymorphisms (RFLPs) [1, 51, 54, 57, 68], amplified fragment length polymorphisms (AFLPs) [10], random amplified polymorphic DNA (RAPDs) [10], and microsatellites [47, 48]. This chapter reviews the present status of the genome relationships of *Avena* species based on molecular genetic and cytogenetic studies.

## OAT GENOMES: AN OVERVIEW

The genus *Avena* contains four different genomes, A, B, C and D, each with a basic chromosome number of seven. The A and C genomes are represented by diploid species, but no diploid species with B or D genomes are known. The B and A genomes occur only in the combination AABB in wild oat species, while D occurs with A and C as AACCCDD, a hexaploid form seen in wild and cultivated oats. The C

genome also occurs in combination with A as AACC, a tetraploid form of wild oats. On the basis of karyotype analyses [11, 65], chromosome pairing affinities [71] and molecular markers [10], five A-genome diploids and two C-genome diploids have been described. Table 1 shows

**Table 1.** Oat genomes identified to date

Ploidy	Species	Genomes
2x	<i>A. strigosa</i> Shreb	AsAs
	<i>A. hirtula</i> Lag.	AsAs
	<i>A. wiestii</i> Steudel	AsAs
	<i>A. hispanica</i>	AsAs
	<i>A. brevis</i> Roth	AsAs
	<i>A. matritensis</i>	AsAs
	<i>A. lusitanica</i>	AsAs
	<i>A. atlantica</i> Baum et Fedak	AsAs
	<i>A. damascena</i> Rajhathy et Baum	AdAd
	<i>A. longiglumis</i> Durieu	AlAl
	<i>A. prostrata</i> Ladizinsky	ApAp
	<i>A. canariensis</i> Baum Rajhathy et Sampson	AcAc
	<i>A. eriantha</i> Dur. ( <i>A. pilosa</i> M.Bieb)	CpCp
	<i>A. clauda</i> Dur.	CpCp
	<i>A. ventricosa</i> Bal. ex Cross.	CvCv
4x	<i>A. barbata</i> Pott ex Link	AABB
	<i>A. vaviloviana</i> (Malz.) Mordí	AABB
	<i>A. abyssinica</i> Hochst	AABB
	<i>A. agadiriana</i> Baum et Fedak	AABB
	<i>A. maroccana</i> Gdgr. ( <i>A. magna</i> Murphy et Terrell)	AACC
	<i>A. murphyi</i> Ladizinsky	AACC
	<i>A. insularis</i>	AACC
6x	<i>A. macrostachya</i> Bal ex Coss. et Dur.	CCCC
	<i>A. sativa</i> L.	AACCDD
	<i>A. byzantina</i> C. Koch	AACCDD
	<i>A. sterilis</i> L.	AACCDD
	<i>A. fatua</i> L.	AACCDD

the oat genomes confirmed to date. All the representative species are annual inbreeders with the exception of *A. macrostachya*, which is an outbreeding perennial autotetraploid [43].

The genome size of oats has been determined by Feulgen microdensitometry (Table 2). In diploid species, it ranges from 4.0 to 5.6 picograms (pg), the A genome being the smallest and the C genome the largest [6]. In tetraploid species it ranges from 8.5 to 9.7 pg, the AABB genome being the smallest and AACC the largest [6]. The genome of the cultivated *A. sativa* (13.2-13.7 pg) is the smallest of the hexaploid cereal species; for example, it is about 21% smaller than that of the cultivated wheat *Triticum aestivum* (15.7-17.3 pg) [6]. One half of the total oat genome occurs as repeat sequences [3]. It is estimated that the flow cytometry [4] reduces the 1C genome of *A. sativa* to only 11.725 pg (equivalent to 11,315 Mb), which is about 33% smaller than that of hexaploid wheat (c.15,966 Megabase) [4]. Thus, the average

**Table 2.** Nuclear DNA content of 16 *Avena* species determined by Feulgen microdensitometry [6]

Species	Genome	DNA Content	
		pg/1C	pg/2C
<i>A. strigosa</i> Shreb	AsAs	4.0 – 5.0	8.0 – 10.0
<i>A. hirtula</i> Lag.	AsAs	4.4 – 4.9	8.8 – 9.8
<i>A. wiestii</i> Steudel	AsAs	4.9 – 5.1	9.8 – 10.3
<i>A. brevis</i> Roth	AsAs	4.5 – 4.7	8.9 – 9.5
<i>A. longiglumis</i> Dur.	AlAl	4.9 – 5.3	9.8 – 10.6
<i>A. eriantha</i> Dur.	CpCp	4.7 – 5.5	9.5 – 11.0
<i>A. clauda</i> Dur.	CpCp	5.3	10.6
<i>A. ventricosa</i> Bal ex Coss.	CvCv	5.6	10.9
<i>A. barbata</i> Pott ex Link	AABB	8.9 – 9.3	17.8 – 18.5
<i>A. vaviloviana</i> Malz.	AABB	8.5 – 9.2	17.0 – 18.4
<i>A. abyssinica</i> Hochst	AABB	8.9 – 9.0	17.9 – 18.0
<i>A. maroccana</i> Gdgr	AACC	9.3 – 9.7	18.6 – 19.4
<i>A. sativa</i> L.	AACCDD	13.2 – 13.7	26.5 – 27.5
<i>A. byzantina</i> C. Koch	AACCDD	13.5 – 13.7	27.1 – 27.4
<i>A. sterilis</i> L.	AACCDD	13.7 – 14.3	27.3 – 28.6
<i>A. fatua</i> L.	AACCDD	12.9 – 14.2	25.7 – 28.3

chromosome size of this wheat is 760 Mb, while the average size of the *A. sativa* chromosomes is 539 Mb. This is comparable to the average size of a *Pisum sativum* (563 Mb) [4] chromosome.

Although the large size of the cultivated oat genome hinders the development of genetic maps with well established linkage groups, a number of maps based on several types of molecular marker have been produced [9, 18, 30, 61, 74]. The most comprehensive hexaploid map was produced using the cross *A. byzantina* cv. Kanota x *A. sativa* cv. Ogle [58, 72]. In its current version it shows a total of 1,166 markers including RFLPs, AFLPs, RAPDs, SSRs, isozymes and seed proteins grouped into 29 linkage groups. Efforts are being made by using approaches such as monosomic analysis, to reduce this number to the 21 expected [14].

## GENOME DIFFERENTIATION AMONG DIPLOID SPECIES

Genome differentiation studies were initially based on descriptions of karyotypes and cytological observations made on interspecific hybrids. Early karyotype studies recognized two basic genomes for the diploid species: the A genome with isobrachial chromosomes, and the C genome with heterobrachial chromosomes [63]. This variation in chromosome morphology has also been demonstrated by C-banding. The chromosomes of A-genome species mainly show telomeric bands, whereas those from the C-genome species are characterized by higher chromatin condensation and the presence of several intercalary bands [11]. Chromosome pairing behaviour at meiosis in the interspecific AC hybrid (represented by *A. strigosa* x *A. eriantha*) involves a high number of unpaired chromosomes and shows partial homology/homeology between the A and C genome chromosomes [46, 56].

This lack of homology has also been borne out by several biochemical and molecular markers. Three systematic approaches have established a very clear separation of the A and C genomes. The first was based on the analysis of biochemical traits such as seed proteins and leaf isozymes. In an extensive gel electrophoresis study of seed proteins, a lack of correlation ( $r=0.04$ ) was found among electrophoretic bands from the A- and C-genome diploid species [33]. In leaf isozyme studies, a group of electrophoretic bands characteristic of the C-genome diploids was absent from the A-genome diploids [8, 67]. The second approach was based on the examination of the number and chromosomal location of ribosomal



DNA loci (18S-5.8S-26S and 5S rDNA). As a rule, 18S-5.8S-26S rDNA loci are found in the nucleolus-organizing regions (NORs). They are cytologically visible by conventional C-banding and Ag-NOR (silver staining of NORs) techniques as secondary constrictions on satellited chromosomes. Using these techniques, two pairs of satellited chromosomes were identified in both A- and C-diploid species, except for *A. ventricosa*, which has only one satellited chromosome pair [11, 65]. FISH and Southern hybridization analyses using heterologous probes have obtained direct information on the differences between ribosomal loci. FISH employing wheat pTa71 (to detect NOR loci) and pTa794 (to detect 5S loci), rDNA probes showed the A-genome diploid species as having two pairs of 5S loci on both arms of one pair of satellited chromosomes, while the C-genome diploid species showed one pair of 5S loci on the long arm of one pair of subtelocentric chromosomes carrying an extra rDNA loci on the same long arm [50]. In Southern analysis using a maize rDNA probe for *Eco*RI-digested DNAs from diploid species, the C-genome diploids were found to have a 10.5-kb *Eco*RI fragment not present in the intergenic spacer (IGS) of the A-genome diploid species [26]. A third approach was based on the isolation of several genome-specific repeat sequences from A-genome diploid species and the use of RAPD and AFLP molecular markers. Four different repeat DNA sequences were isolated from an *A. strigosa* genomic library. As120a satellite DNA was found to be present in A-genome diploid species (with the exception of *A. longiglumis* and *A. damascena*), and absent in C-genome diploid species in experiments involving the hybridization of the pAs120a clone in Southern blots containing *Mun*I-digested genomic DNA from diploid species [51]. Similar information has been reported using two dispersed repeat sequences, As14 and As121 [53] and an LTR (long terminal repeat) fragment of a *Ty1-copia*-retrotransposon [52]. More recently, major clustering differences between the A and C genomes have been detected from phenograms produced with AFLP and RAPD markers. All A-genome diploid taxa were clustered together, while the C-genome diploids formed an outer branch [10].

Structural differences between the A genomes of diploid species have been revealed by the study of genomic relationships through chromosome pairing and fertility. Crosses involving *A. longiglumis* and *A. hirtula*, *A. strigosa* or *A. wiestii* resulted in the failure of chromosome pairing and high levels of hybrid sterility [20, 64, 70]. The cross between

*A. prostrata* and *A. longiglumis* is partially fertile [34]. *A. canariensis* x *A. damascena* hybrids show complete and regular pairing, but they are nonetheless self-sterile [39]. Almost complete chromosome pairing is observed in *A. damascena* x *A. longiglumis* hybrids [39]. All other crosses between A-genome species produce hybrids with irregular chromosome pairing at meiosis [39, 42]. Based on these observations, the genomic symbols As, Ad, Al, Ap and Ac (Table 1) have been suggested to distinguish between the five different karyotypes of the A-genome diploid species [71].

Molecular studies have been one of the major sources of information concerning the divergence of the A-genome diploid species. In autoradiograms of Southern blots of genomic DNAs digested with a single restriction endonuclease and hybridized with either a satellite repeat sequence (As120a) [51], or cDNA clones encoding seed storage proteins and  $\alpha$ -amylase [1], divergence was observed between three endemic species (*A. longiglumis*, *A. damascena* and *A. canariensis*) and the other A-genome diploids. Information on the clustering of RFLP, RAPD and AFLP molecular markers has led to the genomes of *A. nuda* [57], *A. lusitania* and *A. matritensis* [10] being designated as As, although no karyotypic classification is currently available for these species. Based on genetic similarities estimated from RAPDs and RFLPs, Nocelli et al. [57] proposed that the A-genome species be divided into two main groups, the first with the As- and Ap-, and the second with Al-, Ad- and Ac-genome species. This conclusion is supported by studies on the similarity and genome organization of resistance gene analogue sequences (RGAs). The RFLP patterns of *A. strigosa* RGAs in *A. longiglumis*, *A. damascene* and *A. canariensis* show clear differences, both in the number and size of hybridizing bands, with patterns of the *A. strigosa* group of species [23]. This classification, however, contrasts with the genetic similarities estimated by RAPD and AFLP markers reported by Drossou et al. [10]. These authors indicate the Ad-genome species to be closely related to the As-genome species, with the Ap-genome species being the most distant. They also suggest the evolutionary sequence Ap  $\rightarrow$  Al  $\rightarrow$  Ad/Ac  $\rightarrow$  As for the speciation of the A-genome diploids.

The C-genome diploids are represented by two karyotypes, Cp and Cv, corresponding to *A. eriantha*/*A. clauda* and *A. ventricosa*, respectively [11, 65]. Chromosome pairing and molecular markers have revealed structural differences between the Cp and Cv genomes. *A. eriantha* x *A.*

*clauda* hybrids undergo regular meiosis in which seven bivalents are formed. In contrast, hybrids from crosses of *A. ventricosa* with the former show irregular meiosis and are completely sterile [65], reflecting the rearrangements of chromosome structure that differentiate the Cp and Cv genomes. This differentiation is also supported by RAPD and AFLP marker patterns, which show *A. eriantha* and *A. clauda* to be more closely related to each other than to *A. ventricosa* [10].

## GENOME DIFFERENTIATION AMONG TETRAPLOID SPECIES

The tetraploid species can be classified into four groups based on their karyotypes and patterns of chromosome pairing. The first group includes *A. barbata*, *A. vaviloviana* and *A. abyssinica*. These are genetically uniform and possess the AABB genome [65]. Cytological studies of meiotic chromosome in hybrids between AABB and AsAs autotetraploids show the B genome chromosomes to have large segments homologous to those of the As genome [66]. The C-banding patterns of the B genome chromosomes are similar to those of the A genome chromosomes [12]. In addition, GISH experiments employing total *A. strigosa* genomic DNA as a probe have uniformly labelled all 28 chromosomes of *A. barbata* [45] and *A. vaviloviana* [31], reflecting the close relationship between the A and B genomes. In dendrograms produced from AFLP [10] and RGA [23] data, the AABB tetraploids cluster closely with the As-genome diploids. Accordingly, the genomic designation AAA'A' has been suggested for these species. However, FISH experiments employing the As120a A genome-specific satellite sequence as a probe labelled only 14 of the 28 chromosomes of *A. barbata* and *A. vaviloviana* [21]. This shows the presence of two different chromosome sets and supports the AABB genomic designation of these species. This has been confirmed by assigning the satellited chromosomes to individual genomes, using the satellite itself and two ribosomal probes in simultaneous and sequential FISH analyses. Differences between *A. barbata* and *A. vaviloviana* genomes have also been revealed by FISH and Southern blotting techniques [21].

The second group includes *A. agaridiana* from Morocco [5]. Cytological studies of chromosome pairing in triploid hybrids between *A. agaridiana* and A genome diploid species have revealed the chromosomes of *A. agaridiana* to show residual homology with those of the A genome

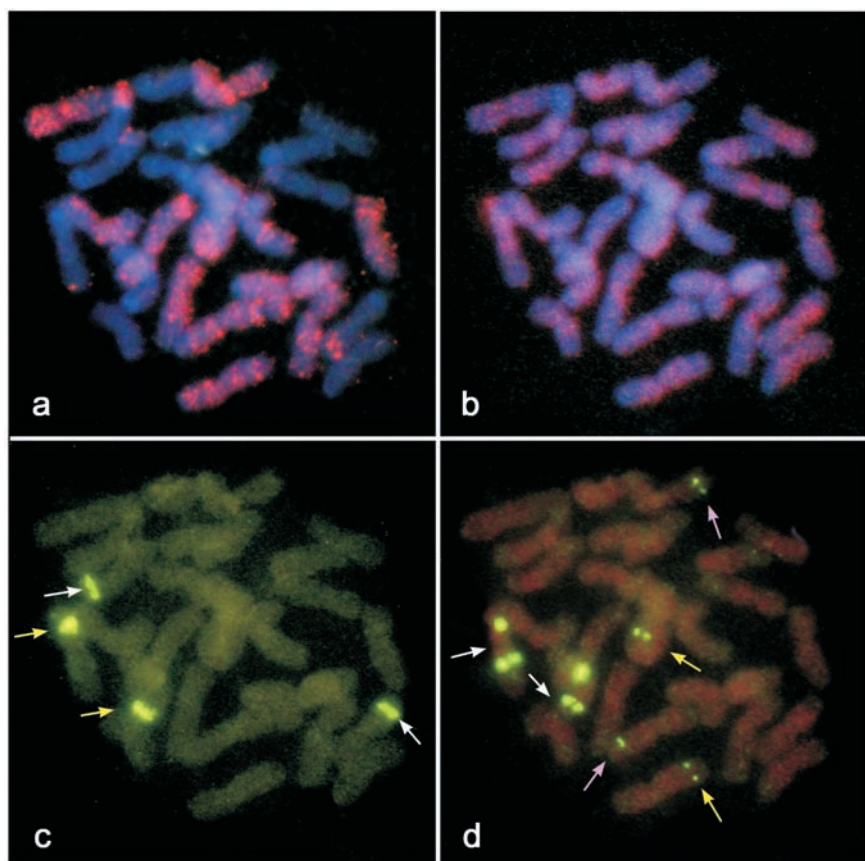
[41]. Chromosome pairing studies in hybrids with *A. barbata* [41] revealed some structural similarities among chromosomes of both species. However, the *A. agaridiana* chromosomes do not closely match those of any of the described diploid or tetraploid species in terms of their arm ratios and C-banding patterns, although their overall C-band appearance resembles that of the A, B and D groups of chromosomes [27]. Consequently, the identity of the genomes remains undefined and requires further study.

The third group of tetraploids includes *A. maroccana* and *A. murphyi*, which have the AACC genome [65]. Cytological studies of chromosome pairing in hybrids of AACC tetraploids and *A. strigosa* indicate the As genome of the diploids to be partially homologous with one of the genomes of the tetraploid species [65]. Isozyme analyses have related *A. eriantha* to *A. maroccana* and *A. murphyi* [8]. The C-banding patterns identified two sets of chromosomes with one or the other of the two heterochromatin distribution patterns described for the chromosomes of A- and C-genome diploids [12]. GISH experiments employing either total *A. strigosa* or *A. eriantha* genomic DNA as a probe [19, 25, 45], and FISH using Am1 (a satellite sequence specific to the C-genome chromosomes isolated from *A. murphyi*) [13], confirmed the AACC genomic constitution of these species. These studies detected that at least seven or eight chromosome pairs in *A. maroccana* and *A. murphyi*, respectively, were involved in intergenomic interchanges between the A and C genomes.

A new tetraploid species, *A. insularis* Ladiz. [37] shares the gross morphological characteristics of the AACC tetraploid species. However, chromosome pairing in hybrids of *A. insularis* and *A. maroccana* showed little affinity between their chromosomes [37]. Similar results were obtained with *A. insularis* x *A. murphyi* hybrids [38]. The C-banding patterns show some structural similarities between the karyotype of *A. insularis* and those of the described AACC tetraploids, but are insufficient to support the unequivocal presence of the A and C genomes in this new tetraploid species [28]. In an effort to clarify this ambiguity, the present report describes the genomic constitution of *A. insularis* as revealed by FISH using probes pAm1 [13] and pAs120a [51]. Also, the genomic designation of each satellited chromosome was achieved by simultaneous FISH and reprobng the same metaphase plates with ribosomal pTa71 [15] and pTa794 [16].

When the rhodamine-labelled pAm1 probe was hybridized, seven pairs of the C-genome chromosomes were successfully labelled (red in Fig. 1a). Surprisingly, when the rhodamine-labelled pAs120a probe was hybridized with the same *A. insularis* metaphases, the 14 chromosome pairs appeared hybridized and showed a dispersed distribution of sequences (red in Fig. 1d). This is the first species studied to show a set of C-genome chromosomes that hybridize with the A-genome-specific As120a sequence in non-translocated areas. However, these results confirm the presence of two genomes in this tetraploid species: a C genome composed of the seven chromosome pairs that hybridized with pAm1, and seven other chromosome pairs initially assigned to the A genome based on hybridization with the pAs120a probe. When FISH was performed on the same metaphase plates with pTa 71 (green in Fig. 1c, to detect 18S-5.8S-26S rDNA loci) or pTa 794 (green in Fig. 1d, to detect 5S rDNA loci), one chromosome pair carrying both NOR and 5S loci, plus another pair carrying only a NOR locus, were detected. In both cases, they belonged to the A genome. Moreover, two chromosome pairs carrying 5S loci were detected (green in Fig. 1d). These were assigned to the C genome.

Both the satellited chromosome pairs of the A genome had terminally located Am1 homologous sequences, indicating the existence of two intergenomic translocations in the formation of *A. insularis*. Taking into account the description of the chromosomes of *A. sativa* after hybridization with the same probes [53], these two pairs of chromosomes should be identified as chromosomes 3D and 13D. Similarly, the 5S rDNA loci detected on the two chromosome pairs of the C genome closely resembled those of *A. sativa* [53] and should be identified as chromosomes 2C and 4C. These are involved in another two intergenomic translocations. Taken together, the FISH results of the present study show a relationship between the C genomes of *A. insularis* and the hexaploid species. However, the initially characterized A genome of *A. insularis* more closely resembles the D genome of the hexaploids than the A genome of these species. Karyotypic evidence [28] and the existence of high chromosomal pairing in hybrids between *A. insularis* and *A. sativa* [38], suggest these two species probably share two genomes. However, since pAs120a failed to hybridize with the C- and D-genome chromosomes of the C genome diploids, the AACCC tetraploids and the AACCCDD hexaploids [51], the C and D genomes of *A. insularis* should be considered modified and perhaps designated C'C'D'D'.



**Fig. 1.** Fluorescent in situ hybridization (FISH) of mitotic metaphase plates of *Avena insularis* (a-d). (a) FISH with the rhodamine-labelled (red) pAm1 probe. (b) The same cell as in (a) shown after FISH with the rhodamine-labelled pAs120a probe (red). (c) The same cell as in (a) and (b) shown after FISH with the digoxigenin-labelled pTa71 (green) probe. Arrows indicate the chromosomes carried NOR loci. (d) The same cell as in (a), (b) and (c) shown after FISH with the digoxigenin-labelled pTa794 (green) probe. Arrows indicate C'-D' and D'-C' intergenomic translocations.

The fourth group of tetraploid species includes *A. macrostachya*, an outbreeding perennial autotetraploid. Chromosome pairing in the hybrids of this species with either *A. sativa* or *A. murphyi* indicates that although there is some homology between the chromosomes of these species and those of *A. macrostachya*, it should be considered as little more than residual [40]. Chromosome pairing in triploid hybrids between this species and either A-genome or C-genome diploid species indicates that



*A. macrostachya* is more related to the C-genome diploids than to the A-genome diploids [40, 44, 60]. The latter's C-banding patterns are similar to those described for the C-genome diploid species [11, 62]. However, with the evidence currently available, the genomic constitution of this species remains doubtful.

## GENOME DIFFERENTIATION AMONG HEXAPLOID SPECIES

The commonly recognized hexaploid species include four that are interfertile: two cultivated species, *A. sativa* and *A. byzantina*, and two wild species, *A. fatua* and *A. sterilis* [36]. Based on chromosome numbers, centromere position and chromosome pairing relationships, it has been proposed that all hexaploids share the same genomic composition of AACCCDD [65]. In recent years, conventional and molecular cytogenetic techniques have been used to identify the three constituent genomes of hexaploid species. C-banding techniques have indicated that both the A and D genomes consist largely of euchromatic chromosomes, whereas the C-genome chromosomes contain largely heterochromatic regions [24, 49]. Fluorescent in situ hybridization with either genomic DNA [7, 19, 25, 45, 73], repeat DNA sequences specific to the C-genome chromosomes [13], or a combination of rDNA genes and cloned repeat DNA sequences [50, 53], indicate that the chromosomes of the D genome bear substantial similarities to those of the A genome. Discrimination among chromosomes of these genomes is possible using an A-genome-specific probe [51]. Together, these studies have confirmed the common genomic composition of the hexaploid species.

Molecular cytogenetic studies have also demonstrated that intergenomic translocations are present in the hexaploid species. Within species, some of these translocations seem to be common to all the cultivars or accessions studied; other translocations would have led to new genotypes. GISH has detected nine intergenomic translocations between the chromosomes of the A/D and C genomes in different cultivars of *A. sativa* [7, 25, 45], eight in accessions of *A. sterilis* [25] and *A. fatua* [73], and five in accessions of *A. byzantina* [25]. The existence of intergenomic translocations specific to a single or reduced number of hexaploid cultivars confirms the importance of these kinds of rearrangement in the evolution of the genus. As an example, FISH employing multiple cloned probes including pAs120a (which hybridizes

exclusively with A-genome chromosomes), pAm1 (which hybridizes exclusively with C-genome chromosomes), pAs121 (which hybridizes exclusively with A- and D-genome chromosomes), pTa71 and pTa794, identified ten intergenomic translocations in the cultivar SunII of *A. sativa* [22]. These were: (i) between the A and C genomes (chromosome pair 5A), (ii) between the C and D genomes (pairs 1C, 2C, 4C, 10C and 16C), and (iii) between the D and C genomes (pairs 9D, 11D, 13D and 14D). The translocation involving chromosomes 10C and 14D differentiate this cultivar from other *A. sativa* cultivars [25, 53].

## PUTATIVE PROGENITORS OF THE HEXAPLOID GENOMES

The evolution of *Avena* genomes has been a complex process involving divergence from a common diploid ancestor, followed by convergence, and subsequently by divergence at the polyploidy level [71].

It has been suggested that the evolution of the AACCCD genome involved two distinct steps. The first was the establishment of the tetraploid (AACC) by the hybridization of two diploid species (AA and CC), followed by a doubling of the chromosome number. This was followed by the hybridization of this tetraploid with a third diploid species, and subsequently by the doubling of chromosomes causing the triploid hybrid to become a hexaploid [43]. The tetraploid species *A. murphyi* was formerly favored as the donor of the AC genome to the hexaploid species. This conclusion was based on chromosome pairing in hybrids between hexaploid and tetraploid oats [32, 35], isozyme analysis [67], and the polymorphism detected using minisatellite and microsatellite sequences [47, 48]. However, the data provided by AFLP and RAPD markers more strongly point to *A. maroccana* [10].

The C-genome diploids have been subdivided into two groups, CpCp and CvCv. Presently there is substantial evidence that *A. eriantha* (CpCp genome) is closely related to the tetraploids and hexaploids. This conclusion was initially based on karyotypes [65] and C-banding [12, 24] analyses. The use of *A. eriantha* DNA in GISH showed the presence of this chromatin in the C-genome chromosomes of tetraploid and hexaploid species [7, 19, 25, 45, 73]. FISH employing the Am1 sequence isolated from *A. murphyi* showed this sequence to be present in *A. eriantha* chromosomes and the C-genome chromosomes of the tetraploid and hexaploid species [13]. Moreover, studies involving molecular



markers such as minisatellites and microsatellites have shown that *A. clauda* (CpCp genome) is closely related to the AC-genome tetraploids and the hexaploids [48]. Nevertheless, two C-genome-specific sequences, AvsC-88 and AvsC-137 cloned from *A. sativa*, have been found in *A. ventricosa* (CvCv genome) and the AC-genome tetraploids and hexaploids [2], indicating that a relationship also exists between these two genomes.

The origin of the A genome in tetraploid and hexaploid species has been a point of controversy. In hexaploid species, initial comparisons of the conventional [65] and C-banding [24, 49] karyotypes of *A. sativa* and diploid *A. strigosa* showed the genome of the latter to very closely match the putative A genome of the hexaploid species. Chromosome pairing in tetraploid hybrids between *A. strigosa* x *A. sativa* was slightly higher than that observed in tetraploid hybrids involving other A-genome diploids [71]. This might indicate greater homology between *A. strigosa* and *A. sativa* than between this species and any other A-genome species. GISH employing *A. strigosa* DNA showed the presence of this chromatin in the A- and D-genome chromosomes [7, 19, 25, 45, 73]. Similarly, FISH employing several repeat sequences isolated from *A. strigosa*, such as dispersed [53] or Ty1-copia-retrotransposon [52, 54] elements, showed hybridization over 14 pairs of chromosomes corresponding to the A and D genomes. These studies, together with the physical position of the 5S ribosomal loci [50], show the A and D genomes of hexaploid species to be highly homologous to the *A. strigosa* genome. However, a satellite sequence, As120a, isolated from *A. strigosa* is able to differentiate between the A and D genomes of hexaploids, and can also distinguish between several A-genome diploid species. The As120a sequence is understood to be a sequence specific to the A genome [51].

*A. canariensis* has also been proposed the most likely progenitor and A-genome donor of the hexaploid species on the basis of genetic similarity data from the analysis of mini- and microsatellite polymorphisms [48]. However, this proposal disagrees with data obtained in Southern experiments which show the As120a sequence comes from the *A. canariensis* genome [51]. Therefore, *A. canariensis* (or some related species) could only be involved in the evolution of the hexaploids as a donor of the D genome.

In tetraploid species, disagreement exists over the implication of *A. strigosa* as the A genome donor. GISH experiments have shown that the

chromosomes of AACC tetraploids contain chromatin of *A. strigosa* [25, 45] as well as various repeat sequences isolated from *A. strigosa* [52, 53, 54]. It has also been demonstrated by Southern experiments that *A. murphyi* contains DNA sequences isolated from *A. sativa* [2, 17]. However, the C-banding patterns of the A-genome chromosomes of the *A. murphyi* and *A. maroccana* species bear little resemblance to those of the *A. strigosa* species [12, 25]. Moreover, only three pairs of A/D-genome chromosomes of hexaploid species bear a close resemblance to the A-genome chromosomes of the two AACC tetraploid species [25]. This is corroborated by the results of a chromosome pairing study involving *A. strigosa* and the AACC-genome tetraploids; these found little evidence to support the presence of the *A. strigosa* genome in the tetraploid species [71]. When the distribution of chromosome markers, such as ribosomal loci and translocated segments, is studied in tetraploid and hexaploid species, a close resemblance is seen between chromosomes 4A and 8A of *A. murphyi* and the corresponding 3D and 13D chromosomes of *A. sativa* [50, 51, 52]. Together, these observations, plus the lack of any in situ hybridization of As120a with the tetraploid species [51], seem to indicate that the so-designated A genome in *A. murphyi* was donated by a diploid species bearing a genome more similar to the present day D genome of the hexaploid species. Therefore, a genomic designation of CCDD for *A. murphyi* is proposed.

Given the evidence currently available, the two above-mentioned steps in the evolution of the hexaploid species should probably be modified. Thus, the first step probably established a tetraploid CCDD (such as *A. murphyi* or a similar species) by the hybridization of a C-genome diploid species (such as *A. eriantha*) and a D-genome diploid species (presumably a diploid species with a modified A genome), followed by a doubling of the chromosome number. The second step involved the hybridization of this tetraploid with an A-genome species such as *A. strigosa*, forming a hexaploid by the doubling of the chromosomes of the resulting triploid hybrid. This new proposal concerning the formation of the hexaploid species is supported by the intergenomic translocations detected in hexaploid species, which appear to have occurred frequently during the evolution of *Avena* [7, 13, 19, 25, 45, 50, 51, 52, 53, 54, 73]. The discrimination of the three genomes of the hexaploids by in situ hybridization with probes specific for either the A-genome or D-genome chromosomes has allowed the number of intergenomic translocations in hexaploids to be determined. Thus, three

C-D and D-C translocations are found in cultivars of *A. sativa*, but so far only one C-A translocation has been detected [21, 53, 54]. Moreover, the two translocations that the cultivar SunII presents in excess also involve the D and C chromosomes. If translocations occurred throughout evolution at a steady rate, the larger number of rearrangements among C and D chromosomes could be interpreted as reflecting a longer “life in common” for these two genomes, rather than for either of these in combination with the A genome. This suggests that a tetraploid CCDD hybridized with *A. strigosa* to give rise to the AACDD hexaploids.

It is interesting to note that none of the molecular reports mentioned in this review include mention of *A. insularis*. Although chromosome affinities between *A. insularis* and *A. sativa* have been described by both meiotic pairing of their hybrids [37] and chromosome marker analysis (this report), the hybridization patterns of the As120a sequence in the C'- and D'-genome chromosomes obtained in the present study indicate that *A. insularis* is more recent than *A. murphyi*. Consequently, *A. insularis* is probably not a candidate progenitor of the hexaploid species.

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# **PLANT GENOME: BIODIVERSITY AND EVOLUTION**

Volume 1, Part D

**Phanerogams (Gymnosperm) and  
(Angiosperm-Monocotyledons)**

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